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# Design and synthesis of estrogen receptor degradation inducer based on a protein knockdown strategy

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ABSTRACT

We designed and synthesized estrogen receptor (ER) degradation inducers 5, 6, and 7, which crosslink the ER and the cellular inhibitor of apoptosis protein 1 (cIAP1). Compounds **5**, **6**, and **7** induced cIAP1-mediated ubiquitylation of ERα resulting in its proteasomal degradation.



Breast cancer is the most common form of cancer in women, and its incidence is increasing year by year. Estrogen receptors (ERs) are often overexpressed in the tissues of breast cancer patients, which promotes the estrogen-dependent proliferation of cancer cells.[1–3] Therefore, ER antagonistic drugs, such as tamoxifen, a non-steroidal selective estrogen receptor modulator, are effective at treating breast cancer.[4,5] Tamoxifen is metabolized by CYP2D6 and CYP3A4 into 4-hydroxytamoxifen, which has 30–100 times more affinity for the estrogen receptor than tamoxifen itself.[6] Tamoxifen is the most frequently prescribed drug for the treatment of all stages of breast cancer,[7] and it is also used to prevent the disease in women who are at high risk of developing breast cancer.[8] However, tamoxifen has agonistic effects on ER in uterus cancer cells and increases the risk of endometrial cancer.[9,10] Furthermore, tamoxifen activates the protein kinase B (Akt) signaling pathway by binding to a particular ER variant, resulting in the inhibition of apoptosis in cancer cells.[11,12]

Recently, we reported a protein knockdown strategy for inducing the degradation of a target protein using the ubiquitin-proteasome system (UPS).[13–18] To degrade proteins of interest, we developed SNIPER (*S*pecific and *N*on-genetic *I*AP-dependent *P*rotein *E*rasers), hybrid molecules composed of bestatin (BS), an inhibitor of the cellular apoptosis protein 1 (cIAP1), and a ligand for the target protein.[13–15] These molecules cross-link cIAP1 and the target protein to induce ubiquitylation and subsequent proteasomal degradation of the target protein.

In this study, we applied this methodology to the selective degradation of ERα in breast cancer cells. We designed SNIPER(ER) hybrid molecules **5**, **6**, and **7**, which induce the selective poly-ubiquitylation of the ER to cause proteasomal degradation. Molecules **5**, **6**, and **7** contain two biologically active scaffolds: one is a tamoxifen derivative, which is used to bind to the ER, and the other is a BS moiety, which binds to cIAP1 to induce the ubiquitylation and subsequent proteasomal degradation of the target protein (Fig. 1). Molecules with different length linkers **5**, **6**, and **7** were designed based on the X-ray structure (PDB ID: 3ERT) of the complex formed between 4-hydroxytamoxifen and ERα,[19] and BS was ligated to the dimethylamino moiety of 4-hydroxytamoxifen (red circle in Fig. 2a) via an alkyl linker (Fig. 2b).

The ligated molecules **5**, **6**, and **7** were synthesized as follows (Scheme 1). First, (*E*/*Z*)-endoxifen (**1**)[20,21] was condensed with several acids to afford amides **2**, **3**, and **4**. After deprotection of one of the *N*-Boc protecting groups of **2**, **3**, and **4**, the generated amines were reacted with *N*-Boc bestatin,[11] and the subsequent deprotection of another of the molecules' *N*-Boc protecting groups gave ligated compounds **5**, **6**, and **7**, respectively.[22]

Then, we evaluated the effects of compounds **5**, **6**, and **7** on ERα protein degradation in MCF-7 breast cancer cells by examining the treatment dose (Fig. 3).[23] The protein expression of ERα was increased by treatment with (*E*/*Z*)-endoxifen (lane 3), which was consistent with the findings of a previous report demonstrating that (*E*/*Z*)-endoxifen induced the accumulation of ERα protein in

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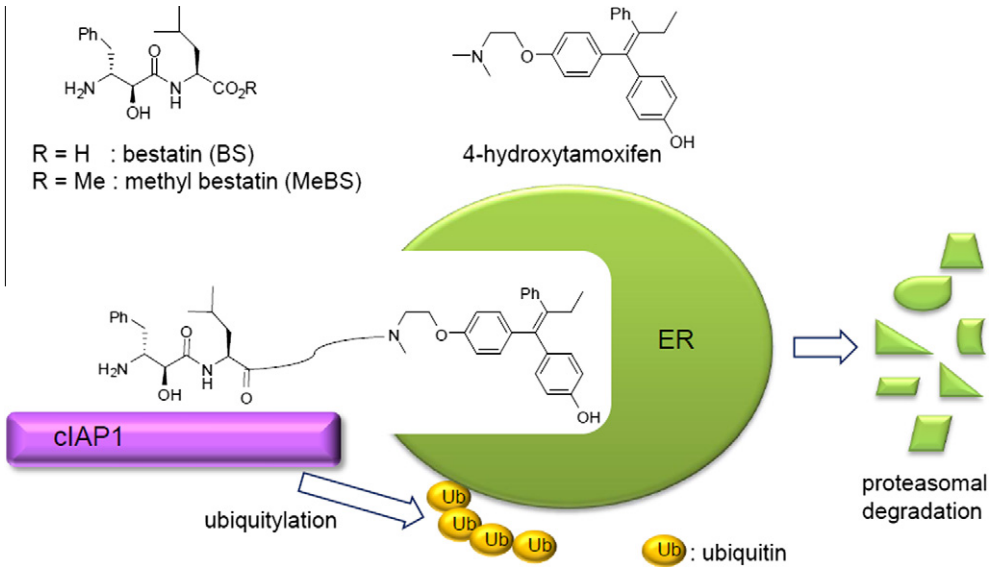



**Figure 1.** Estrogen receptor degradation strategy using a ligated hybrid molecule.

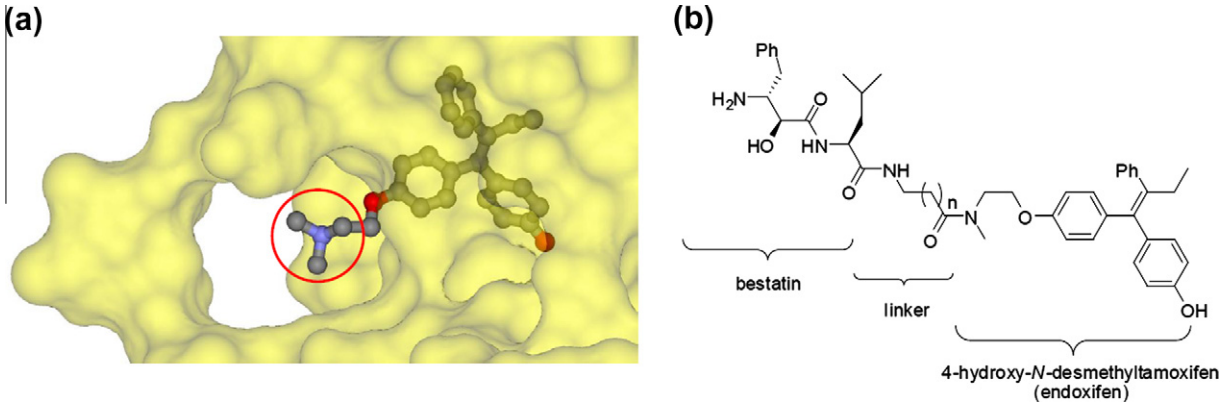
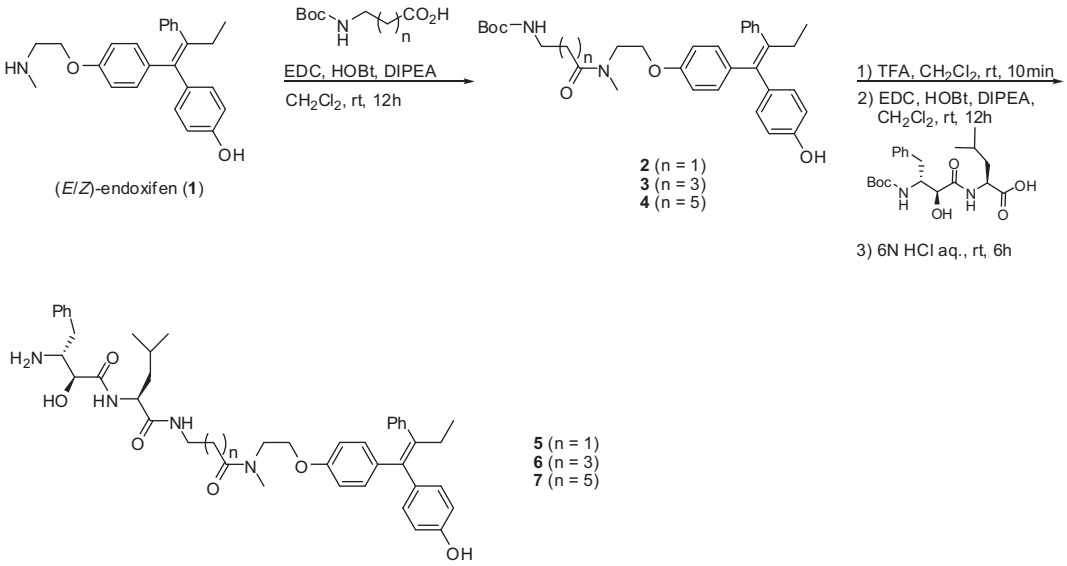



**Figure 2.** (a) X-ray structure of the complex formed between 4-hydroxytamoxifen and ERα(3ERT). (b) Design of the SNIPER(ER) ER degradation inducer.




**Scheme 1.** Synthesis of ligated compounds **5**, **6**, and **7**.

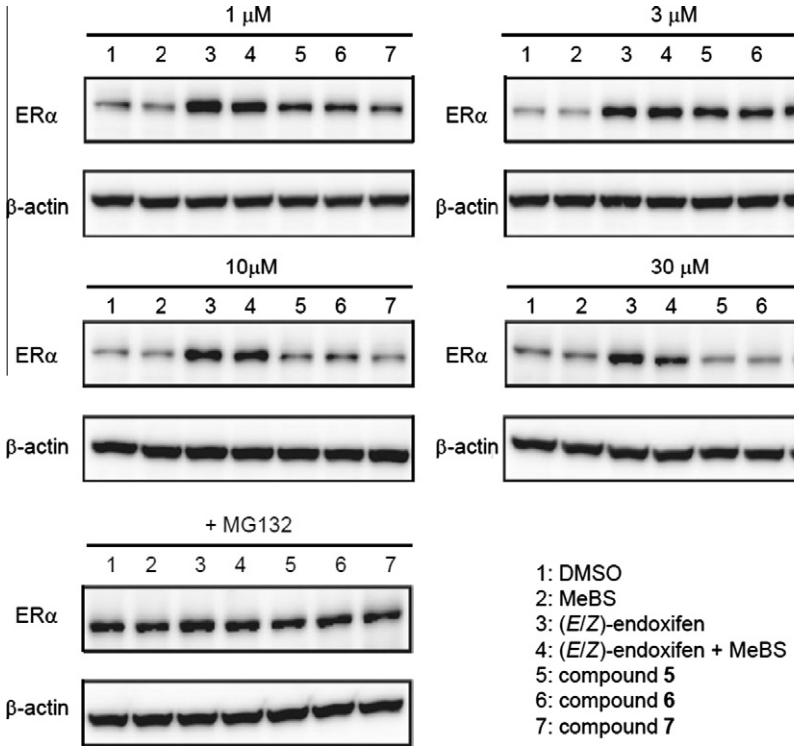



**Figure 3.** Dose-dependent ERα degradation responses induced by compounds **5**, **6**, and **7** and the effects of a protease inhibitor on these responses.

MCF-7 cells.[24] Compared with (*E*/*Z*)-endoxifen (lane 3), a reduced level of ERα was observed in the cells treated with 10 or 30 μM of compound **5**, **6**, or **7** (lanes 5, 6, and 7, respectively), and no apparent differences in the activities of them were observed during the 6 h study period. On the other hand, the ERα level was not altered by the combined use of (*E*/*Z*)-endoxifen and methyl bestatin (MeBS)[25] (lane 4). These results suggest that (*E*/*Z*)-endoxifen conjugated with BS as a single molecule (at a concentration of greater than 10 μM) is required for the efficient degradation of the ERα protein. Furthermore, the suppressive effects of compounds **5**, **6**, and **7** on ERα were blocked by the addition of a proteasome inhibitor, MG132, indicating that compounds **5**, **6**, and **7** induced the proteasomal degradation of ERα.

In summary, we used a protein knockdown method for the selective degradation of ERα and synthesized ERα degradation inducers **5**, **6**, and **7**, which form crosslinks between ERα and cIAP1. Compounds **5**, **6**, and **7** were able to induce cIAP1-mediated ubiquitylation and hence induce the proteasomal degradation of ERα. These molecules are novel candidates for therapeutic agents against breast cancer, and further derivatization of these molecules is currently underway.

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23. MCF-7 cells were treated with the test compounds at the indicated concentrations in the presence or absence of 10 μM of MG132 for 6 h, and then the cells were collected and extracted with lysis buffer (1% SDS, 0.1 M Tris–HCl (pH 7.0), 10% glycerol) and boiled for 10 min. Protein concentrations were determined by the BCA method, and equal amounts of protein lysate were separated by SDS–PAGE, transferred to a PVDF membrane, and Western blotted using the following antibodies: anti-human ERα mouse monoclonal antibody (Santa Cruz), anti-β-actin mouse monoclonal antibody (SIGMA), and anti-human cIAP1 goat polyclonal antibody (R&D systems).
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25. MeBS interacts with the BIR3 domain of cIAP1 and induces the auto-ubiquitylation of cIAP-1, resulting in the proteasomal degradation of cIAP1: See Ref. 16.