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Jostling for Position: Optimizing Linker Location in the Design of Estrogen Receptor-Targeting PROTACs

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Estrogen receptor-α (ER) antagonists have been widely used for breast cancer therapy. Despite initial responsiveness, hormone-sensitive ER-positive cancer cells eventually develop resistance to ER antagonists. It has been shown that in most of these resistant tumor cells, the ER is expressed and continues to regulate tumor growth. Recent studies indicate that tamoxifen initially acts as an antagonist, but later functions as an ER agonist, promoting tumor growth. This suggests that targeted ER degradation may provide an effective therapeutic approach for breast cancers, even those that are resistant to conventional therapies. With this in mind, we previously demonstrated that proteolysis targeting chimeras (PROTACs) effectively induce degradation of the ER as a proof-of-concept experiment. Herein we further refined the PROTAC approach to target the ER for degradation. The ER-targeting PROTACs are composed of an estradiol on one end and a hypoxia-inducing factor 1α (HIF-1α)-derived synthetic pentapeptide on the other. The pentapeptide is recognized by an E3 ubiquitin ligase called the von Hippel Lindau tumor suppressor protein (pVHL), thereby recruiting the ER to this E3 ligase for ubiquitination and degradation. Specifically, the pentapeptide is attached at three different locations on estradiol to generate three different PROTAC types. With the pentapeptide linked through the C7α position of estradiol, the resulting PROTAC shows the most effective ER degradation and highest affinity for the estrogen receptor. This result provides an opportunity to develop a novel type of ER antagonist that may overcome the resistance of breast tumors to conventional drugs such as tamoxifen and fulvestrant (Faslodex).

Introduction

Breast cancer is the most common form of malignant disease in women worldwide. The majority of breast tumors, about two thirds of those initially diagnosed, are estrogen receptor-α (ER)-positive. In most cases the ER plays a significant role in the stimulation and growth of these breast tumors.[1,2] For example, the protein level of the ER is elevated in pre-malignant and malignant breast lesions relative to normal tissue.[1] In addition, the clear correlation between ER-positive breast tumors and their response to anti-estrogen therapy has been demonstrated.[3]

Most ER-positive tumors initially respond well to anti-estrogens, which block the site at which estrogen can bind, thereby halting the growth of cancer cells. Tamoxifen, a non-steroid selective ER modulator (SERM), is an anti-estrogen therapeutic which has been widely used for more than 20 years in all stages of ER-positive breast cancers.[4,5] Specifically, tamoxifen induces a conformational change of the ER that blocks the receptor's function.[6] Another example is fulvestrant (Faslodex),[7,8] which is a pure steroidal anti-estrogen approved for the treatment of post-menopausal women with hormone-sensitive, advanced, or metastatic breast cancers. Like tamoxifen, fulvestrant competitively binds the ER and blocks ER-promoted cell proliferation. Whereas tamoxifen causes accumulation of the ER, fulvestrant, like ER-targeting PROTAC molecules, induces selective proteasome-mediated degradation of the ER and therefore does not exhibit the agonistic effects commonly associated with SERMs.[9] Unfortunately, despite the relative safety and significant anti-neoplastic activities of these anti-estrogens, most initially responsive breast tumors acquire resistance.[10–14] As such, the major challenge is to develop novel therapeutic agents that not only inhibit the growth of hormone-sensitive breast tumors, but also prevent the development of drug resistance.

Although the mechanism by which ER-positive cancer cells acquire drug resistance is not clearly understood, it is unlikely that any single mechanism or gene confers anti-estrogen resistance. Given that less than 25% of tumors which recur following treatment with fulvestrant or tamoxifen lack the ER,[15,16] ER loss does not seem to be the major mechanism that drives acquired resistance. It has also been shown that a loss of anti-estrogen responsiveness by initially sensitive tumors is unlikely due to mutations or deletion of the ER gene.[1,14] In fact, most tumors at recurrence retain levels of ER

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expression that would define them as ER positive.[17–21] In addition, the ER appears to maintain the ability to continuously regulate tumor growth in most anti-estrogen-resistant tumors.[20] It has been suggested that one likely cause of tamoxifen resistance is the ability of tumors to switch from recognizing an anti-estrogen as an antagonist to recognizing it as an agonist, perhaps through differential co-regulator recruitment to the ER.[22–26] For fulvestrant, a conformational change in the ER is suggested to affect the binding of co-regulator(s) and to influence the stability of the ER, thereby causing its rapid degradation. Additionally, fulvestrant causes a loss of the ER protein without affecting ER mRNA levels.[27,28] Consistent with this observation, it has been reported that resistance of human breast cancer cells to fulvestrant is not associated with a general loss of ER expression or lack of estrogen responsiveness.[29] Taken together, it seems that tamoxifen and fulvestrant maintain their ER binding affinity despite the loss of their antiproliferative or ER-antagonistic effects, and that the ER continues to regulate the growth of resistant ER-positive breast cancer cells. Consequently, a small molecule that targets the ER for degradation, regardless of changes that induce drug resistance, may prove useful as an alternative therapeutic option for ER-positive breast cancer therapy.

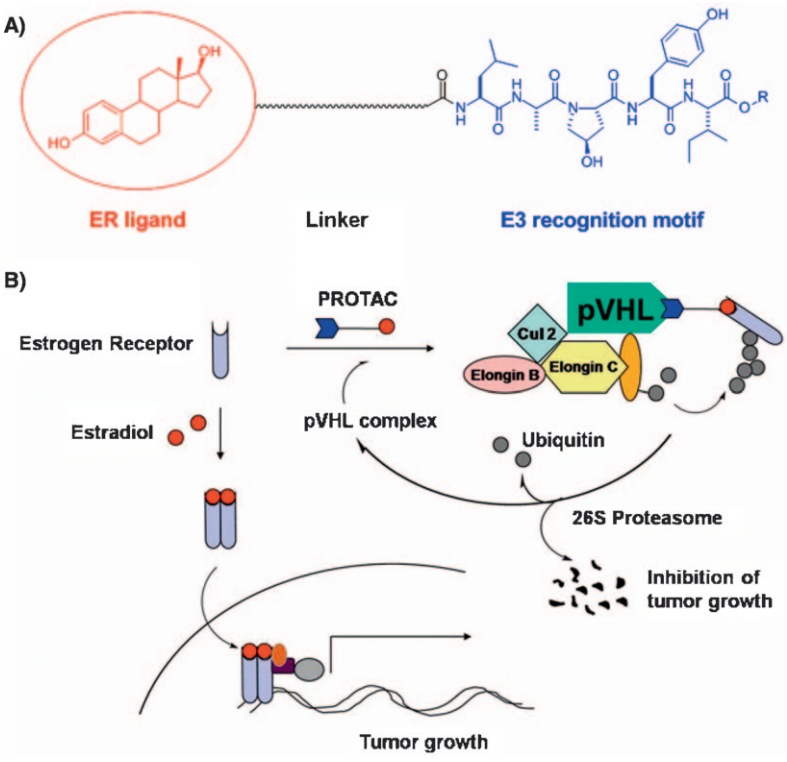


Figure 1. PROTAC-induced targeted degradation of the ER. A) The PROTAC is composed of three components: an E3 ligase recognition motif, linker, and ligand (in this case, targeting the ER). B) The PROTAC recruits the ER to the pVHL E3 ligase complex for ubiquitination and subsequent degradation by the 26S proteasome; this degradation of the ER should decrease the amount of dimerized ER bound to estradiol available to drive gene transcription, a major factor in the growth and survival of breast cancers.

Toward this end, we used a novel small-molecule-based technology termed *PROteolysis TArgeting Chimeras* (PROTACs) to target the ER for degradation at the posttranslational level. PROTAC molecules are composed of three major components (Figure 1 A): a small-molecule ligand for a targeted protein, an E3 ubiquitin ligase recognition motif, and a linker. For the E3 ligase recognition domain, a 12-mer polypeptide containing two phosphoserines (derived from IκBα) was initially used as a proof of concept.[30] However, the poor bioavailability of the 12-mer PROTAC necessitates microinjection for delivery, hence limiting its use as a molecular probe. To circumvent this, an alternative E3 recognition motif was required, so HIF-1α polypeptides were developed. A pentapeptide was found by our research group and others to be sufficient for HIF-1α-based PROTACs to cross cell membranes and to effectively degrade target proteins.[31–33]

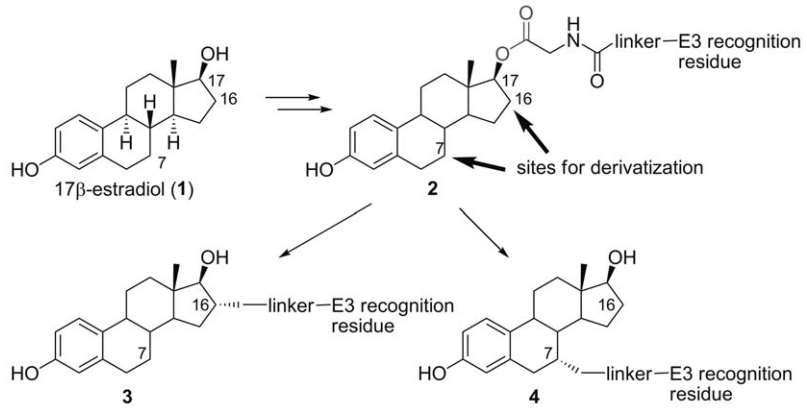
Previously, we developed PROTACs that target the ER by using estradiol as the ligand and the optimized pentapeptide as the E3 recognition motif. These proof-of-concept PROTACs used an ester bond to attach the linker moiety to the O17 position of estradiol. A significant drawback to this attachment site is its vulnerability to esterases, which would inactivate the PROTAC as well as release estradiol. Another critical issue with the O17-linked PROTAC was the loss of the 17-OH group, which is reported to be important for ER binding of E2.[34] Therefore, E2-based PROTACs that can spare the 17-OH group are desirable for the design of improved PROTACs that target the ER.

Other potential positions for derivation from 17β-estradiol have been reported. A geldanamycin-tagged estradiol compound derivatized at the C16 position on estradiol was reported to maintain its interaction with the ER.[35,36] Similarly, derivatization at the C7α position of estradiol has been shown to preserve its binding affinity with the ER.[7,8,37] For instance, fulvestrant has a high ER binding affinity, and it is an E2 derivative with a long hydrocarbon tail at the C7α position. Thus, we endeavored to create a more stable linker by attaching the pentapeptide at the C16 and C7 positions of estradiol to prepare PROTACs (Scheme 1). Herein we report the rational design and optimization of PROTACs that target the ER for ubiquitination and degradation. The information contained herein also provides an example for PROTAC design that will produce molecules with superior effects in cell-based systems.

Results and Discussion

PROTAC synthesis

The parent ER-targeting PROTAC **2** was synthesized as described.[38] In addition, PROTAC **2** was deprotected at the C terminus of the pentapeptide to give PROTAC **2***, using the same procedure as described below (**13** and **14**). To synthesize PROTAC **3**, a "free amine handle" was introduced at the C16α

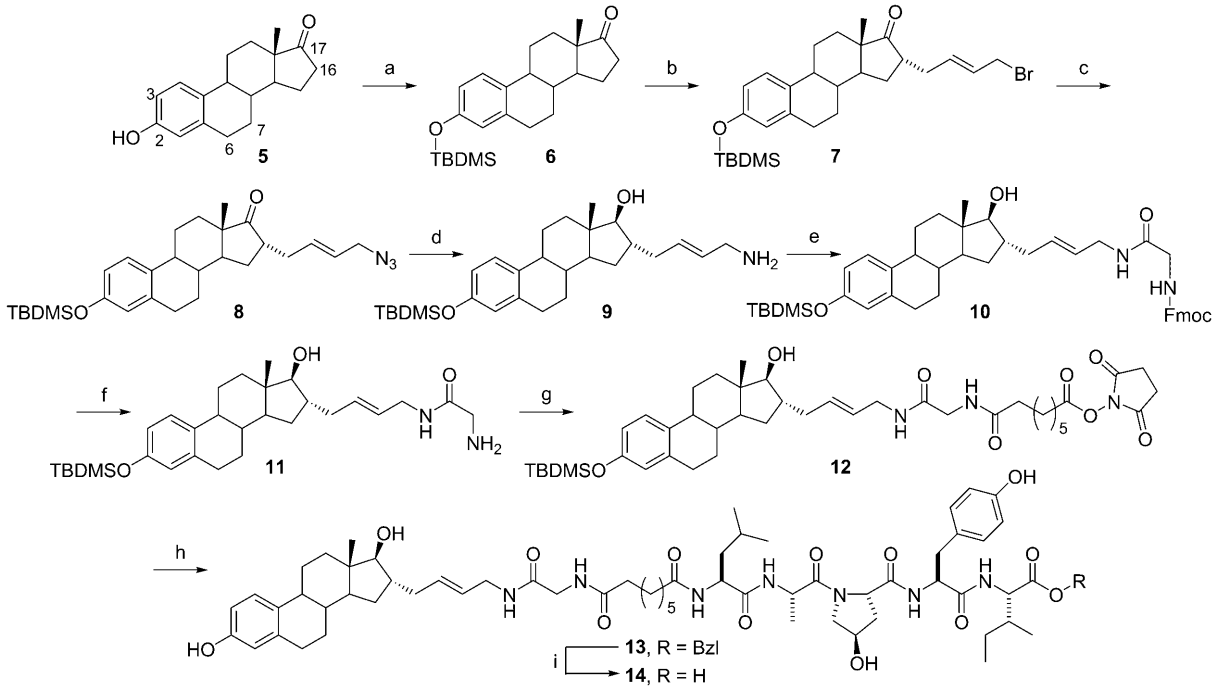


Scheme 1. Synthetic strategy for next-generation ER-targeting PROTACs. Attaching the linker by a carbon–carbon bond should produce PROTACs with higher ER binding affinity and stability in living cells than the parent ER-targeting PROTAC **2**.

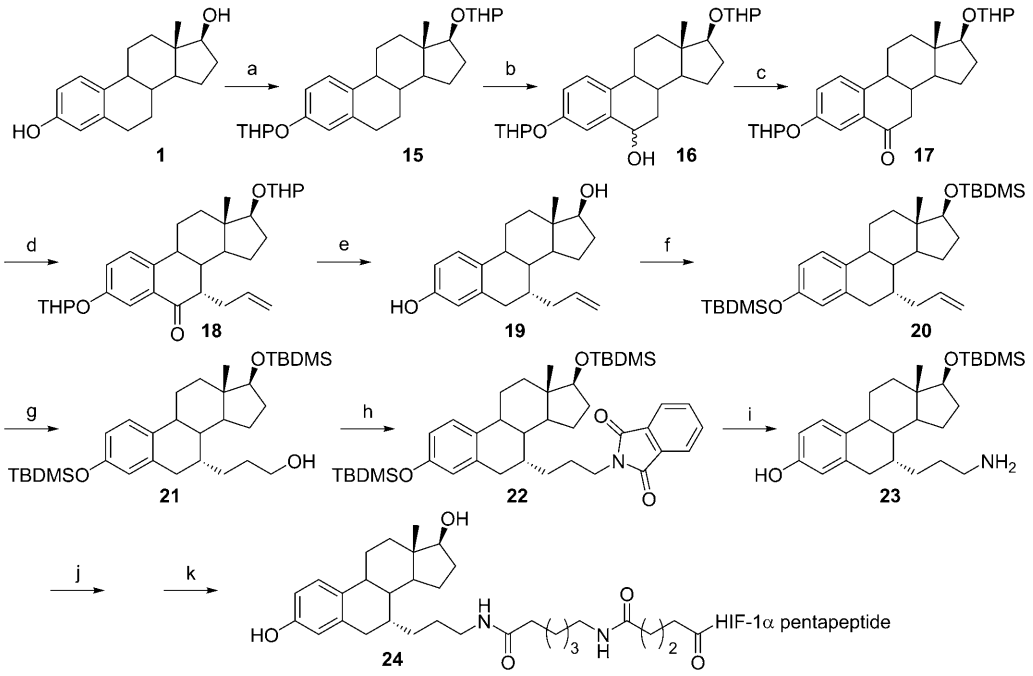
position of E2 (17β-estradiol) via a carbon–carbon linkage, by following a procedure similar to that previously reported (Scheme 2).[36,39,40] First, estrone **5** was treated with *tert*-butyldimethylsilyl chloride (TBDMSCl) to yield compound **6**. Alkylation at the C16 position of **6** was carried out by using lithium diisopropylamide (LDA) and 1,4-dibromo-2-butene. The resulting alkyl bromo residue was converted into the azido compound **8** by treatment with sodium azide. Sequential reduction of the keto and azide groups with sodium borohydride and lithium aluminum hydride yielded the free amine "handle", **9**. The handle was further extended with glycine to give **11**, which was activated to the succinimidyl ester **12** by using disuccinimidyl suberate (DSS). Compound **12** was finally coupled with the HIF-1α pentapeptide, and the TBDMS group was deprotected with tetrabutylammonium fluoride (TBAF) to yield PROTAC **13**. The benzyl group of PROTAC **13** was removed by hydrogenolysis to obtain PROTAC **14**.

As was the case for PROTAC **13**, the most critical step for the synthesis of PROTAC **24** is the introduction of a primary amine "handle" at the C7α position of estradiol (Scheme 3). A procedure similar to that reported by Skaddan and Katzenellenbogen[41,42] was followed to introduce this handle. As a first step, tetrahydropyran (THP)-protected **15** was synthesized by following a procedure similar to that previously described.[43–45]

Deprotonation at the C6 position under "superbase" conditions followed by treatment with trimethyl borate yielded an intermediate borate ester, which was oxidized with hydrogen peroxide to provide **16** as epimers. Further oxidation of **16** with pyridinium chlorochromate (PCC) afforded the keto functionality at the C6 position in **17**. Treatment of **17** with potassium *tert*-butoxide and allyl iodide afforded **18**. Simultaneous deprotection of the THP groups and the C6 keto functionality



Scheme 2. Synthesis of the C16-derivatized estradiol-based PROTACs: a) TBDMSCl, imidazole, 99%; b) LDA, $C_4H_6Br_2$, THF, 45%; c) NaN_3, THF, DMSO, H_2O, 96%; d) 1. $NaBH_4$, MeOH; 2. $LiAlH_4$, THF, 35%; e) Fmoc-Gly-OH, HBTU, DIPEA, CH_2Cl_2, 47%; f) 20% piperidine in DMF, 67%; g) DSS, DMF, 38%; h) 1. HIF-1α pentapeptide, DMAP, DMF; 2. TBAF, THF, 47%; i) H_2, Pd/C, EtOAc, MeOH, 89%.



Scheme 3. Synthesis of an estradiol containing an amine functional group at the C7 position: a) DHP/TsOH, 94%; b) 1. LiDAKOR; 2. $B(OMe)_3$; 3. H_2O_2, 66%; c) PCC, 80%; d) 1. KO*t*Bu, C_3H_5I, 2. NaOMe, 36%; e) Et_3SiH, $BF_3{\cdot}Et_2O$, 90%; f) TBDMSCl, imidazole, 85%; g) 1. 9-BBN; 2. H_2O_2, KOH, 66%; h) PPh_3, DIAD, phthalimide, 83%; i) NH_2NH_2, 76%; j) disuccinimidyl glutarate, 37%; k) 1. HIF-1α pentapeptide; 2. TFA, 25% over two steps. HIF-1α pentapeptide = Leu-Ala-Pro^{OH}-Tyr-Ile-Bzl.

was carried out with triethylsilane and boron trifluoride diethyl etherate to give **19**. The free OH groups were re-protected with TBDMS groups to give **20**, which was then treated under hydroboration–oxidation conditions to produce alcohol **21**. Treatment of **21** with phthalimide under Mitsunobu conditions followed by hydrazinolysis provided the primary amine "handle" **23**. Compound **23** was further extended and activated using DSS. Sequential coupling of HIF-1α pentapeptide and deprotection of TBDMS groups yielded PROTAC **24**.

Biological characterization of PROTACs

PROTACs **2**, **2***, **13**, **14**, and **24** were tested for their ability to degrade the endogenous ER in MCF7 breast cancer cells. After incubation with PROTACs for 48 h, their effects on ER protein levels were evaluated by western blotting analysis and immunofluorescence.

Previous reports indicated that the O17-linked PROTAC **2** would degrade the ER in a proteasome-dependent manner.[31,32,38] Our initial data confirmed this finding (Figure 2A) while demonstrating that the C-terminal-protected PROTAC **2** is superior to the deprotected PROTAC **2***. A major drawback to these compounds is their susceptibility to endogenous esterases, so we moved forward with the testing of carbon-based linkages to confirm the results from these proof-of-concept PROTACs. A similar trend in ER degradation was observed for the C16α-based PROTACs **13** and **14**, with the protected pentapeptide providing superior degradation (Figure 2B). The C7α-based PROTAC **24** showed a similar ability to degrade the ER when compared with the C16α-based PROTAC **13** (Figure 2B).

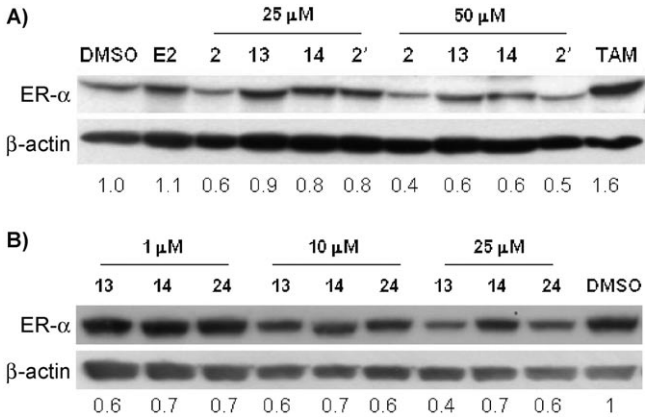


Figure 2. C-terminal protection of the HIF-1α pentapeptide provides improved degradation of the ER by PROTACs linked at any position on estradiol. A) O17 (**2** and **2***) and C16 (**13** and **14**) derivatives of E2-based PROTACs with the benzyl-protected HIF-1α pentapeptide (**2** and **13**) are more effective in degrading the endogenous ER than unprotected PROTACs (**2*** and **14**). B) A similar pattern was observed for the C7 derivative of E2-based PROTAC, as the benzyl-protected compounds **13** and **24** showed greater ER degradation than the deprotected compound **14**.

Next, we further confirmed the PROTAC-mediated degradation of the ER in MCF7 cells by immunofluorescence studies. Estradiol (E2) was used as a positive control. E2 induces proteasome-mediated degradation of the ER as a result of transcriptional activation, resulting in cell proliferation. ER degradation by E2 was blocked by co-treatment with epoxomicin, a natural

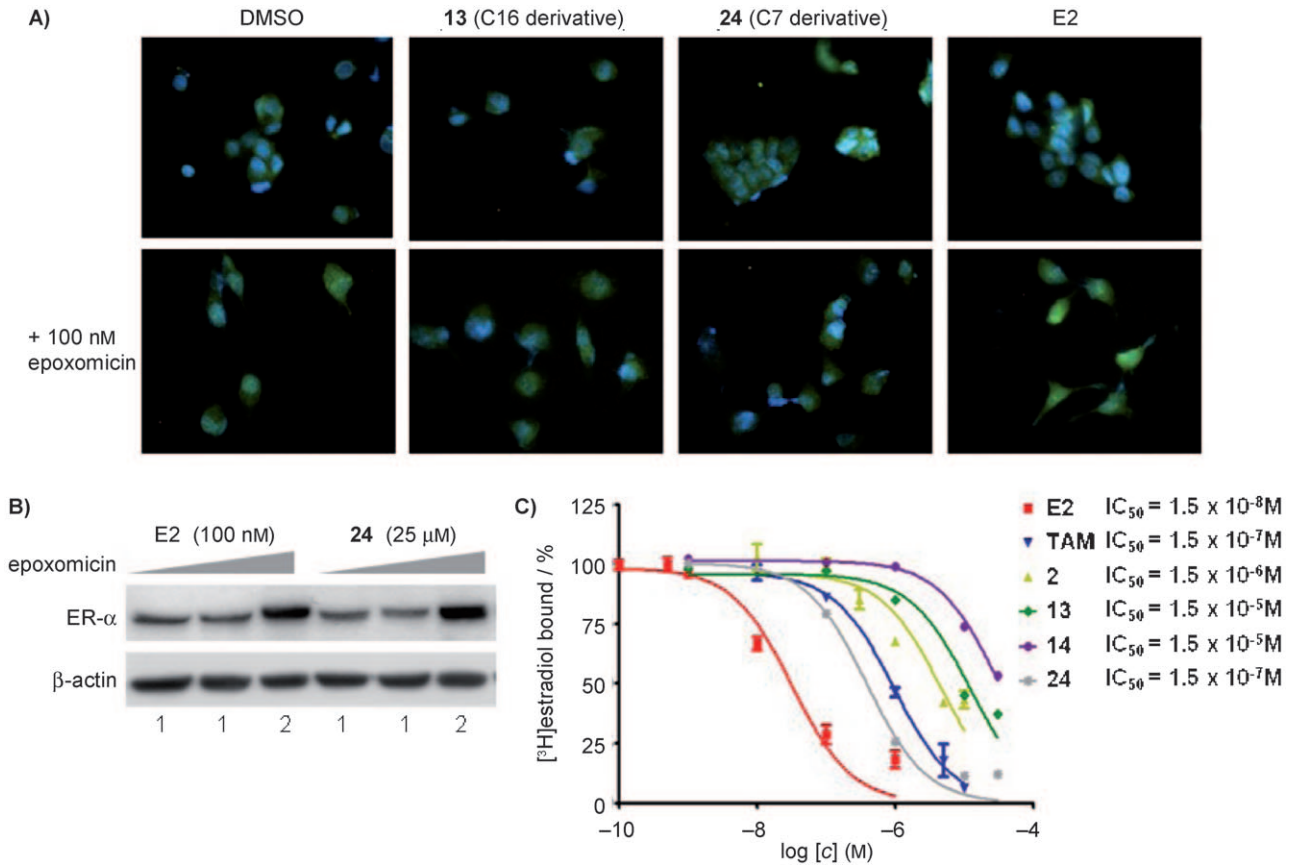


Figure 3. PROTACs bind to the ER and cause its proteasome-mediated degradation. A) Immunofluorescence images of MCF7 cells after 48 h treatment with 25 μM PROTACs, 10 nM E2, or DMSO vehicle. The ER is visualized by conjugation with FITC (green), and DAPI staining (blue) indicates the nucleus. Co-treatment with epoxomicin causes accumulation of the ER. B) Western blot data demonstrating the proteasome dependence of PROTAC-mediated ER degradation. C) Competitive binding assay indicating that C7 is a superior position for PROTAC derivatization due to its enhanced binding to the ER relative to the O17- and C16α-based PROTACs.

product proteasome-specific inhibitor. Compared with DMSO control, the attenuation of ER signal (green) was observed with the treatment of **13** and **24** (Figure 3A, top row). These compounds showed an accumulation of the green signal when co-treated with epoxomicin, which was also observed in control/epoxomicin treated cells (Figure 3A, bottom row). This result was confirmed by western blotting using **24** and E2 (Figure 3B). To validate the superior position for PROTAC linkage, a competitive ligand binding affinity assay was performed. These in vitro data indicate that the C7α-based PROTAC **24** has the highest affinity of the E2-based PROTAC compounds tested, superior even to tamoxifen. The binding data for the C16α-based PROTACs **13** and **14** also support the data from Figure 2 which indicates that benzyl protection of the C terminus of the pentapeptide gives a superior compound. Because a PROTAC with a higher binding affinity for the target protein should induce optimal ubiquitination and subsequent degradation by the proteasome, further optimization of the PROTAC approach for the ER should use the C7α linkage.

Conclusions

The ubiquitin–proteasome degradation pathway is a destructive, irreversible process and controls many important biological processes such as cell-cycle progression, differentiation, and inflammation. We have shown previously that the cellular ubiquitination machinery can be hijacked by a PROTAC containing a pentapeptide that is recognized by the E3 ligase pVHL, and that a PROTAC containing a ligand of a target protein induces ubiquitination and degradation of that protein in living cells.[31,38,46] This "chemical knockout" approach provides flexible spatial and temporal control, which is critical to dissect complex signaling pathways in cells.

As many diseases are driven by the expression of a few proteins, a potential therapeutic strategy is to remove these essential proteins. Small interfering RNAs (siRNAs), which knock down the expression of a gene of interest by destroying its mRNAs, have drawn considerable attention as a potential therapeutic approach.[47,48] However, this siRNA knock-down strategy has some inherent problems,[47–49] such as off-target effects, sequence-independent effects, activation of unrelated signaling pathways, and drug delivery. The PROTAC approach does not appear to suffer these same limitations and thus may

provide an additional strategy to treat diseases by destroying disease-promoting proteins.

As ER-positive breast cancers require ER-promoted cell proliferation, the current therapeutic paradigm relies on the blockage of this hormone-mediated cell growth. Thus, a strategy which allows selective degradation of the ER could provide a valuable and novel therapeutic option for many breast cancer patients. Additionally, most breast cancers that develop resistance to current treatments retain functional ERs. Optimized PROTACs, which induce the degradation of the ER, may thus provide an additional therapeutic option for the treatment of tamoxifen- or fulvestrant-resistant breast tumors.

In summary, we have shown that a chimeric small molecule induces the proteasome-dependent degradation of the ER in living cells. Whether the targeted degradation of ER is useful in treating hormone-sensitive or anti-estrogen-resistant breast tumors remains to be determined. Generally, PROTACs should be optimized by determining a position for derivatization that retains a maximal binding affinity. Additionally, pentapeptide-based PROTACs benefit from C-terminal protection of the peptide regardless of the linker position. Finally, the small-molecule strategy reported herein presents a generic approach to target any cancer-promoting protein for degradation.

Experimental Section

Chemistry

All reagents were purchased from Aldrich. PROTAC **2** was synthesized by following a procedure similar to that previously reported.[50] Epoxomicin was synthesized as previously reported.[51] See the Supporting Information for more detailed information on the chemical syntheses.

Biological assays

Reagents: Fetal bovine serum (FBS), RPMI 1640, phenol-red-free RPMI, conjugated secondary antibody Alexa Fluor 488 (FITC), antibiotics, Hank's balanced salt solution (HBSS), goat serum, Prolong Gold antifade with DAPI, recombinant human estrogen receptors (ER-α), and trypsin–EDTA were purchased from Invitrogen (Carlsbad, CA, USA). 17β-Estradiol, tamoxifen, Kodak XAR film, NaCl, Nonidet-P40 (NP40), protease inhibitors cocktail, Tween-20, ethanol, bovine serum albumin (BSA), and 2× Laemmli sample buffer were obtained from Sigma–Aldrich (St. Louis, MO, USA). Charcoal–dextran-treated FBS was purchased from Hyclone (Logan, UT, USA). Anti-ER antibodies were acquired from Santa Cruz Biochemical (Santa Cruz, CA, USA) for western blotting, and Abcam (Cambridge, MA, USA) for immunofluorescence, while the anti-β-actin antibody was purchased from Novus Biologicals (Littleton, CO, USA). The anti-mouse IgG–horseradish peroxidase (HRP) conjugate was obtained from Zymed Laboratories (South San Francisco, CA, USA). Anti-rabbit IgG–HRP and enhanced chemiluminescence (ECL) detection reagents were acquired from GE Healthcare (Piscataway, NJ, USA). Protein Assay Dye Reagent Concentrate, Tris·HCl, Triton X-100, sodium dodecyl sulfate (SDS), and PVDF membranes were purchased from Bio-Rad (Hercules, CA, USA). Dimethyl sulfoxide (DMSO) and KCl were obtained from EMD (Darmstadt, Germany), while the CellTiter 96 Aqueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI, USA). Paraformaldehyde (PFA) was purchased from Fisher Scientific, while monobasic and dibasic potassium phosphate were acquired from Mallinckrodt Baker (Phillipsburg, NJ, USA). Antibody Dilutant with Background Reducing Components was purchased from DAKO (Glostrup, Denmark), while [6,7-^{3}H]17β-estradiol was purchased from Amersham Biosciences (Buckinghamshire, UK).

Cell culture: The MCF7 human breast cancer cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). MCF7 cells were maintained in RPMI 1640 medium containing FBS (10% *v*/*v*), penicillin (100 U mL^{-1}), and streptomycin (100 ug mL^{-1}) (Gibco, Carlsbad, CA, USA). All experiments were performed when the cells were 70% confluent and had been maintained in 5% (*v*/*v*) charcoal–dextran-treated FBS RPMI with antibiotics for at least 24 h. Compounds were treated in a DMSO vehicle at the appropriate dilutions for 48 h unless noted otherwise.

Western blotting: Whole-cell lysates were prepared by incubating cells in non-denaturing lysis buffer (50 mM Tris·HCl, 150 mM NaCl, 1% NP40, 1% Triton X-100, and 1% protease inhibitor cocktail) on ice for 1 h. Cells were then centrifuged (14000 rpm, microliter rotor 24×1.5/2 mL, Biofuge Primo R) with supernatants collected and subjected to protein assay by the Bradford method. The sample was mixed with an equal volume of 2× Laemmli sample buffer and heated in boiling water for 10 min. Equal protein concentrations of the samples were subjected to 10% SDS polyacrylamide gel electrophoresis and blotted onto a PVDF membrane. After blocking, the membranes were incubated overnight at 4 °C in primary antibody and for 1 h at room temperature with secondary antibody. Antibody binding was detected using ECL and film. All membranes were then re-probed with mouse anti-β-actin to ensure equal protein loading.

Cell viability assay by MTS: MCF7 cells were plated at a density of 5×10^3 cells per well in 96-well plates in RPMI 1640 with 10% FBS and left overnight at 37 °C. The media was changed to 5% charcoal RPMI for 24 h prior to the addition of compounds. The proliferation rate of the cells was determined after 48 h by using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay according to the supplier's instructions. Absorbance was measured at λ 490 nm on a microplate reader using the KC4 program. IC_{50} values were obtained from at least triplicate results via a sigmoid dose–response curve using nonlinear regression to a logarithmic function (GraphPad Prism software, San Diego, CA, USA).

ER binding affinity assay: Competitive ligand binding assays were performed according to the manufacturer's protocol (Invitrogen). Purified human recombinant ER (10 nM) was added to ^{3}H-labeled estradiol (20 nM) and the indicated concentrations of estradiol or PROTACs. After incubation for 2 h at room temperature or overnight at 4 °C, a 50% hydroxyapatite slurry was added to bind the receptor–ligand complex. The sample was centrifuged (14000 rpm, microliter rotor 24×1.5/2 mL, Biofuge Primo R), and the pellet resuspended for analysis of tritium activity by scintillation counting. The percent specific binding was calculated, and IC_{50} values were obtained using one-site competition curve analyses in GraphPad Prism. Relative binding affinity (RBA) was calculated by the following equation: $RBA = (IC_{50\ E2}/IC_{50\ sample})\times100$.

Immunofluorescence: Cover slips were sterilized with ethanol and UV light exposure. MCF7 cells were added directly to the cover slip and allowed to attach for 24 h. The media was then changed to phenol-red-free RPMI with 5% charcoal–dextran-treated FBS until treatment with compounds. Compounds were diluted in the phenol-red-free media and treated as before. Cells were then fixed with 4% PFA, washed with phosphate-buffered saline (PBS), and

permeabilized with 0.2% Triton X-100 in PBS. Between all subsequent steps, cover slips were washed in PBST (PBS with 0.05% Tween-20). Cover slips were blocked in 10% goat serum with 0.1% BSA in PBST. Next, primary antibody was added in the antibody dilutant directly to the cover slip, then secondary antibody was added in the same way. Prolong Gold with DAPI was added to clean slides to mount the cover slips, and the mounted slides were allowed to dry overnight. After drying, the cover slips were rimmed with clear nail polish and visualized on an inverted fluorescence microscope (Nikon Ti-U) with NIS Element Research image analysis software.

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