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Impact of linker length on the activity of PROTACs

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Abstract

Conventional genetic approaches have provided a powerful tool in the study of proteins. However, these techniques often preclude selective manipulation of temporal and spatial protein functions, which is crucial for the investigation of dynamic cellular processes. To overcome these limitations, a small molecule-based novel technology termed “PROteolysis TARGETing ChimeraS (PROTACs)” has been developed, targeting proteins for degradation at the post-translational level. Despite the promising potential of PROTACs to serve as molecular probes of complex signaling pathways, their design has not been generalized for broad application. Here, we present the first generalized approach for PROTAC design by fine-tuning the distance between the two participating partner proteins, the E3 ubiquitin ligase and the target protein. As such, we took a chemical approach to create estrogen receptor (ER)- targeting PROTACs with varying linker lengths and the loss of the ER in cultured cells was monitored via western blot and fluorometric analyses. We found a significant effect of chain length on PROTAC efficacy, and in this case, the optimum distance between the E3 recognition motif and the ligand was a 16 atom chain length. The information gathered from this experiment may offer a generalizable PROTAC design strategy to further the expansion of the PROTAC toolbox, opening new possibilities for the broad application of the PROTAC strategy in the study of multiple signaling pathways.

Introduction

Small molecule modulators of protein function are finding increasing use as probes of biological systems due to the temporal and spatial control they afford, as they interfere with protein function at the post-translational level. Conversely, traditional genetic approaches use tools (such as gene knockout or RNAi) that knockout or knock-down proteins at the DNA or RNA level, respectively. While these genetic methods have provided useful insights into protein functions, they are limited by such factors as compensatory responses and delivery problems.¹ Furthermore, the lack of tight spatial and temporal control in these approaches hampers the dissection of protein functions in complex signaling pathways. Rational drug design and the screening of libraries containing random small molecules or natural products are some of the tactics that have been employed to identify and develop small molecules which lack these weaknesses.² Typically, the lead small molecule modulators identified by these methods bind target proteins and block their function.

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The ubiquitin-proteasome system (UPS) is a major intracellular protein degradation system and is the principle conduit for protein turnover in all eukaryotic cells. Ubiquitin-dependent proteolysis involves the assembly of an ubiquitin chain on a substrate, which is subsequently targeted by the 26S proteasome for degradation.³⁻⁴ While three enzyme families are involved in the poly-ubiquitination process, the E3 ubiquitin ligases appear to be the primary source of substrate specificity in the ubiquitination cascade. For example, E3 ubiquitin ligases pVHL and MDM2 recruit hypoxia inducing factor-1 (HIF-1 and p53, respectively, to ubiquitination complex for polyubiquitination and subsequent degradation by the 26S proteasome.⁵

Exploiting the interaction between the E3 ubiquitin ligase and its substrate, a novel class of molecular probes termed PROTACs (Proteolysis Targeting Chimerias) has been developed.⁶ PROTACs are designed to recruit a target protein to an E3 ubiquitin ligase complex for poly-ubiquitination and degradation by the proteasome (Fig. 1). Unlike conventional chemical inhibitors, PROTACs do not simply inhibit protein functions; they destroy targeted proteins. The chimeric PROTAC molecule is comprised of three parts: a small peptide residue for E3 ubiquitin ligase recognition, a ligand that can be recognized by a targeted protein, and a linker that connects these two moieties (Fig. 1A). Thus far, a dodecapeptide derived from I B⁷ or pentapeptide derived from HIF-1⁸⁻⁹ have been used as an E3 ubiquitin ligase recognition motif. The 12 amino acid-peptide derived from I B₂, a negative regulator of NF B, is sufficient to bind the mammalian F box protein -TRCP/E3RS, a member of the heterotetrameric Skp1-Cullin-F box (SCF) E3 ubiquitin ligase complex.¹⁰ Meanwhile, the pentapeptide from HIF-1 is the minimum recognition domain for the von Hippel-Lindau tumor suppressor protein (pVHL),⁸ part of the VBC-Cul2 E3 ubiquitin ligase complex. Using these peptide motifs several PROTACs have been successfully developed.¹¹ Proteins targeted by these PROTACs include methionine aminopeptidase 2 (MetAP-2),⁷ the estrogen receptor (ER),^{8, 12-13} the androgen receptor (AR),¹² the aryl hydrocarbon receptor (AHR),¹⁴⁻¹⁵ FK506 binding protein (FKBP),⁹ and cellular retinoic acid-binding proteins (CRABPs).¹⁶ Recently, PROTACs have been investigated as potential therapeutic agents that target disease-promoting proteins for degradation.¹⁷⁻¹⁸

While the PROTAC containing an E3 ligase recognizing pentapeptide residue has been shown to be cell-permeable and effective at inducing the degradation of target proteins^{8-9, 13}, its bioavailability and permeability still need to be addressed for the broad application of the PROTAC approach. In this regard, a polyarginine tail was attached to PROTACs to enhance cell permeability.¹² In addition, Crews' group created an all small-molecule PROTAC to provide high bioavailability and cell permeability by replacing the E3 ligase recognizing peptide residue with nutlin, a non-peptidic ligand for MDM2.¹⁹ Furthermore, natural product geldanamycin (GM)-based chimeric small molecules have been shown to induce degradation of hormone receptors, which normally exist as a complex resistant to proteasomal proteolysis.²⁰⁻²² Given that geldanamycin is known to be a heat shock protein 90 (HSP90) antagonist,²³ this geldanamycin-based degradation appears to be limited to proteins associated with HSP. Despite this limitation, geldanamycin-based molecular tools offer an additional option to specifically target HSP90-associated proteins for degradation.

Contrary to this breadth of modifications to the E3 ligase recognition motif, the other two moieties of PROTACs have received much less attention regarding their optimization for activity. For the protein ligand, typically known synthetic small molecules or the natural ligands have been used.¹¹ Examples include estradiol for the ER, dihydrotestosterone (DHT) for the AR, fumagillin for MetAP-2, and AP21998 for FKBP. Unlike the small molecule ligands, the length of the linker has been randomly selected for PROTACs, varying from 12-carbon to over 20-carbon in length.^{8-9, 12-13} As the concentration of PROTACs currently

required to induce protein degradation is insufficient for broad applicability, optimization of the linker length could afford more potent PROTACs. An optimal linker will allow for maximal interaction between the two moieties (target protein and E3 ligase) resulting in efficient ubiquitination of the target protein (Fig. 2) and its ultimate degradation.

Given that PROTACs possess advantages inherent in both small molecule modulators (spatial & temporal control) and genetic tools (protein knock-down), the PROTAC technology holds great promise as a molecular tool. However, PROTACs are currently prepared on a case by case basis, without a general design strategy for optimum PROTACs. To investigate the impact of linker length, we chose the ER-targeting PROTAC as a model system (Fig. 2). In addition to serving as a facile model system, ER-targeting PROTACs may potentially provide an additional option for the treatment of hormone-sensitive, ER-positive breast cancer.¹² Given that the effect of linker length has never been systematically explored for PROTAC action, in this report we detail the effects of linker length on PROTAC activity. We envision that the information reported herein can be applied to the general design of PROTACs targeting other proteins with known ligands.

Results

ER-targeting PROTACs (detailed procedures are available in the supporting materials)

ICI 182,780 (Faslodex®) is a clinically available ER antagonist with high ER binding affinity. Structurally, it is an E2 derivative with a long hydrocarbon tail at the C-7 position. Therefore, we choose to attach the HIF-1 pentapeptide (E3 ligase recognition motif) at the C-7 position of E2 using varying linker lengths. Specifically, THP-protected **2** was synthesized following a procedure similar to that previously described.^{24–26} Deprotonation at the C-6 position under “superbase” conditions followed by treatment with trimethyl borate yielded an intermediate borate ester which was oxidized using hydrogen peroxide to provide **3** as epimers. Further oxidation of **3** with PCC afforded the keto functionality at the C-6 position in **4**. Treatment of **4** with potassium tert-butoxide (KOt-Bu) and allyl iodide afforded **5**. Simultaneous deprotection of the THP groups and the C-6 keto functionality was accomplished using triethylsilane (Et₃SiH) and boron trifluoride diethyl etherate (BF₃·Et₂O) to give **6**. The free OH groups were reprotected with *tert*-butyldimethylsilyl (TBDMS) to give **7**, which was then treated under hydroboration-oxidation conditions to produce the alcohol, **8**. Treatment of **8** with phthalimide under Mitsunobu conditions followed by hydrazinolysis provided the primary amine “handle”, **10** (Scheme 1).

The amine **10** was used as an intermediate in the synthesis of PROTACs with varying linker lengths (Scheme 2). The amine handle was further extended via amide bond formation with different alkyl carboxylic acids and/or disuccinimidyl alkyl esters (see supporting information).

The resulting monosuccinimidyl alkyl ester was then coupled to the N-terminus of the E3 ligase recognizing pentapeptide to yield **11–15**. Unlike compounds **11–15**, the free amine **10** was also attached to the C-terminus of the pentapeptide to yield **16**. This compound was used to investigate the impact of coupling the linker to C-terminus (**16**) vs N-terminus (**12**) of the pentapeptide. A negative control PROTAC, **17** in which the hydroxyproline of HIF-1 pentapeptide was replaced with norleucine was also synthesized. This compound was used to confirm the loss of activity when binding to pVHL was abrogated.

ER Degradation Assays

PROTACs were tested for their ability to degrade the endogenous ER- in MCF7 breast cancer cells. Initial evaluation of the PROTACs via western blotting analysis was performed

after 48 hour treatments. First, we explored whether the position at which the linker was attached to the pentapeptide was significant. Compounds **12** and **16** have the same linker length and, as shown in Fig. 3A, the N-terminal attachment of the linker to the pentapeptide provides superior ER degradation and thus was maintained in all subsequent experiments. Next, we examined the impact of linker length on N-terminal coupled PROTACs. At the highest concentration examined (50 μ M), all PROTACs but **15** showed significant ER degradation (Fig. 3B). However, examination of ER degradation at lower doses (Fig. 3B & C) showed a preference for PROTACs with hydrocarbon chain lengths less than 16 (**11–13**).

Since PROTACs **11–13** were determined to be superior to **14–16** in terms of ER degradation, we examined these three compounds at even lower doses which suggested that **13** degraded the ER most effectively (data not shown). To confirm this, we next examined the efficacy of two of these shorter PROTACs (**12** and **13**) over time. As seen in Fig. 3D, the ER degradation seen was stable between 48 and 72 hours. Importantly, when the treatment time was further extended and included a media change containing PROTACs (Fig. 3E), **13**, which has a 16 atom linker, proved to be superior to **12**, the 12 atom linker PROTAC.

Next, we wanted to validate that the degradation of the ER is due to the activity of PROTACs and not other factors. As shown in Fig. 4A, treatment with epoxomicin, a proteasome inhibitor, resulted in the accumulation of the ER, demonstrating that ER degradation by **13** is proteasome-dependent. It should be noted that tamoxifen has been shown to accumulate the ER and thus, was used as a positive control. We then investigated whether the degradation of the ER is mediated by the pVHL E3 ubiquitin ligase. To do this, we generated a chemical mutant, **17**, by replacing the critical hydroxyproline in the HIF-1 pentapeptide with norleucine. Given that the hydroxyproline residue of HIF-1 pentapeptide motif is required for recognition by the E3 ligase for ubiquitination, its replacement should render the PROTAC inactive. As shown in Fig 4C, **17** is unable to degrade the ER. However, **12**, which differs only by the hydroxyproline, effectively causes ER degradation. Further, immunofluorescence data clearly shows that the interaction between the E3 ligase and its recognition peptide residue from the PROTAC is required for the degradation of the ER and that epoxomicin can block this ER degradation (Fig. 4C). Taken together, these results demonstrate that the PROTACs degrade the ER in a proteasome- and E3 ligase-dependent manner.

PROTAC Efficacy: Cytotoxicity and Binding Affinity

PROTAC-mediated degradation of the ER requires the PROTAC to interact with the pVHL E3 ligase as well as the ER. To more clearly demonstrate that PROTACs can associate with the ER, an in vitro binding affinity study was performed. As shown in Fig. 5A, the results indicated that all PROTACs have a similar binding affinity for the ER, suggesting the linker length does not influence ER binding affinity. Additionally, PROTACs **12** and **16** have nearly identical binding affinities, indicating the position on the pentapeptide to which the linker is coupled has no effect on ER binding.

Finally, we determined the viability of MCF7 cells to examine whether the degradation of the ER is correlated with the inhibition of cell growth. As MCF7 breast cancer cells are dependent on the ER for cell proliferation, we hypothesized that effective PROTACs would inhibit cell proliferation and eventually cause cell death. As shown in Fig. 5B, PROTAC **13**, which showed high ER degradation activity, was the most effective in inhibiting the proliferation of MCF7 cells after a 48 hour treatment. Taken together, these data indicate the importance of optimizing the length of the linker to produce PROTACs with maximal activity. In the case of the ER, a PROTAC linker length of 16 atoms (compound **13**) seems to be ideal (Fig. 5C).

Discussion

In recent years, the PROTAC technology has drawn considerable interest for the production of chemical probes as well as potential therapeutic molecules.^{17–18} This technology provides a knock-down of protein at the post-translational level, giving highly flexible temporal and spatial control when compared to traditional genetic approaches. Although PROTACs have been successfully developed to target a number of proteins, no general PROTAC design strategy has been proposed. The ligand for the target protein (synthetic or natural product) and E3 recognition residue (eg., HIF-1 pentapeptide) may be predetermined in the design of PROTACs. However, the distance between the two moieties which is optimal to promote efficient ubiquitination may need to be determined on a case by case basis. Indeed, our studies suggest that the length of the linker is critical for the activity of PROTACs. Additionally, the direction of the pentapeptide with respect to the ligand may be a generally important quality, as we found significantly superior protein degradation with N-terminal coupled PROTACs. With the parameters described herein as a starting point, a more straightforward methodology to target other proteins with the PROTAC technology is expected.

Compared to conventional antagonists, PROTACs confer several advantages. For example, PROTAC action is not dependent on active-site inhibition or a protein-protein interaction motif to achieve antagonistic effects. Thus, any binding site unrelated to protein function may be targeted for the design of PROTACs, provided that there are lysines for ubiquitination on the surface of the target proteins. This, in principle, permits the targeting of almost any protein in the cytosolic area, providing a ‘chemical knockout’ of protein function.

PROTACs may provide a notable advantage for the treatment of certain diseases over conventional antagonist drugs. For example, PROTACs may be used to treat recurring breast cancer after initial treatment with tamoxifen or faslodex. It has been reported that tamoxifen initially acts as antagonist in hormone-dependent breast cancer cells but later the cancer cells use it as an agonist, promoting proliferation of tamoxifen-resistant cancer cells. Similarly, the development of faslodex-resistant cancer cells has been a major clinical concern in the treatment of breast cancer. It is important to note that these resistant cancer cells are typically still dependent on the ER for proliferation. In principle, PROTACs that target the ER for degradation may be effective in halting proliferation of these tamoxifen or faslodex-resistant cells. In summary, these experiments suggest that there could be a general strategy to prepare optimum PROTACs by appropriately spacing and orienting the two partner proteins, the ligand and the E3 ubiquitin ligase.

Experimental Section

Biological 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- and ER- and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). 17 β -estradiol, tamoxifen, Kodak XAR film, Sodium Chloride, Nonidet-P40 (NP40), Protease inhibitors cocktail, TWEEN® 20, Ethanol, Bovine Serum Albumin (BSA), and 2X Laemmi sample buffer were obtained from Sigma Aldrich (St. Louis, MO). Charcoal-dextran treated FBS was purchased from Hyclone Co. (Logan, UT). Anti-ER antibodies were acquired from Santa Cruz (Santa Cruz, CA) for western blotting and Abcam (Cambridge, MA) for immunofluorescence while the anti-beta actin antibody was purchased from Novus Biologicals Inc. (Littleton, CO). Anti-mouse IgG horseradish peroxidase was obtained from

Zymed Laboratories (South San Francisco, CA). The anti-rabbit IgG horseradish peroxidase and enhanced chemiluminescence detection reagents (ECL) were acquired from GE Healthcare (Piscataway, NJ). Protein Assay Dye Reagent Concentrate, Tris-Chloride, Triton X-100, Sodium Dodecyl Sulfate (SDS), and PVDF membranes were purchased from Bio-Rad (Hercules, CA). Methylsulfoxide (DMSO) and potassium chloride were obtained from EMD (Darmstadt, Germany) while the CellTiter 96 Aqueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI). Para-formaldehyde (PFA) was purchased from Fisher Scientific while potassium phosphate monobasic and potassium phosphate dibasic were acquired from Mallinckrodt Baker (Phillipsburg NJ). Antibody Dilutant w/ Background Reducing Components was purchased from DAKO (Glostrup, Denmark) while [6, 7-³H 17] oestradiol was purchased from Amersham Biosciences (Buckinghamshire, United Kingdom).

Cell Culture

The MCF7 human breast cancer cell line was purchased from the American Type Culture Collection (Manassas, VA). MCF7 cells were maintained in RPMI 1640 (Gibco, Carlsbad, CA) medium containing 10 % (v/v) FBS (Gibco, Carlsbad, CA) and 100 U/mL penicillin-100 ug/mL streptomycin (Gibco, Carlsbad, CA). 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 hours. Compounds were treated in a DMSO vehicle at the appropriate dilutions for 48 hours unless noted otherwise.

Western Blotting

Whole cell lysates were prepared by incubating cells in nondenaturing lysis buffer (50mM Tris-Cl, 150mM NaCl, 1% NP40, 1% Triton X-100, and 1% protease inhibitor cocktail) on ice for 1 hour. Cells were then centrifuged with supernatants collected and subjected to protein assay via method of Bradford. The sample was mixed with an equal volume of 2X Laemmi sample buffer and heated in boiling water for 10 min. Equal protein concentrations of sample were subjected to a 10% SDS polyacrylamide gel, electrophoresed, and blotted onto a PVDF membrane. After blocking, the membranes were incubated overnight at 4°C in primary antibody and for 1h at room temperature with secondary antibody. Antibody binding was detected using ECL and film. All membranes were then reprobed with mouse anti-beta actin to ensure equal protein loading. The intensities of the bands on western blot films were quantified using volumetric densitometry (Quantity One, Bio-Rad). The estrogen receptor-alpha values were normalized to -actin and DMSO was arbitrarily assigned a value of 100% for comparison purposes. The results were graphed in GraphPad Prism (San Diego, USA) with means and standard deviations calculated from at least two independent experiments.

Cell Viability Assay by MTS

MCF7 cells were plated at a density of 5×10^3 cells per 96 well plates in RPMI 1640 with 10 % FBS and left overnight. The media was changed to 5% Charcoal RPMI for 24 hours prior to the addition of compounds. The proliferation rate of the cells was determined after 48 hours by using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay according to the supplier's instruction. Absorbance was measured at 490nm on a microplate reader using the KC4 program. In Graph Pad Prism (San Diego, CA), IC₅₀ values were obtained from at least triplicate results via a sigmoid dose-response curve using a nonlinear regression to a logarithmic function.

ER Binding Affinity Assay

Competitive ligand binding assays were performed according to the manufacturer's protocol (Invitrogen). 10nM of purified human recombinant estrogen receptor was added to 20nM [³H]-labeled estradiol and the indicated concentrations of estradiol or PROTACs. After an incubation (2 hr at room temperature or overnight at 4°C), a 50% hydroxy apatite slurry was added to bind the receptor/ligand complex. The sample was centrifuged and the pellet resuspended for analysis of tritium activity using scintillation counting. The percent specific binding affinity was calculated and IC₅₀ values were obtained using one site competition curve analyses provided by the Graph Pad Prism. Relative binding affinity (RBA) was calculated using the following equation $RBA = \{IC_{50}(E2)/IC_{50}(\text{sample})\} \times 100$.

Immunofluorescence

Coverslips were sterilized with ethanol and UV light exposure. MCF7 cells were added directly to the dish and allowed to attach for 24 hours. 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 detailed previously. Cells were then fixed with 4% PFA, washed with Phosphate Buffered Saline (PBS), and permeabilized with 0.2% Triton-X in PBS. Between all subsequent steps coverslips were washed in PBST (PBS with 0.05% Tween-20). Blocking in 10% goat serum with 0.1% BSA in PBST was performed at 37°C for 1 hour or 4°C overnight. Primary antibody was added in the antibody dilutant directly to the coverslip, incubated at 37°C for one hour, then secondary antibody was added in the same way with incubation for 30 minutes. Prolong Gold with DAPI was added to clean slides to mount the coverslips. The mounted slides were allowed to dry overnight. After drying, the coverslips were rimmed with clear nail polish and visualized on an inverted fluorescence microscope (Nikon Ti-U microscope) with NIS Element Research image analysis software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PROTACs	PROteolysis Targeting ChimeraS
ER	estrogen receptor
UPS	ubiquitin-proteasome system
HIF-1	hypoxia inducing factor-1
SCF	Skp1-Cullin-F box
pVHL	von Hippel-Lindau tumor suppressor protein
MetAP-2	methionine aminopeptidase 2
AR	androgen receptor

AHR	aryl hydrocarbon receptor
CRABPs	cellular retinoic acid-binding proteins

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Fig. 1. PROTAC-induced targeted degradation of protein. **A.** The PROTAC is composed of three components: an E3 ligase recognition motif, a ligand for target protein, and a linker. **B.** The PROTAC recruits targeted proteins to an E3 ubiquitin ligase for ubiquitination and subsequent degradation by the 26S proteasome.

Fig. 2.
The proposed mode of PROTAC binding with the ER and the pVHL.

Fig. 3.

ER degradation induced by PROTACs varies with linker length. **A.** Attachment of the linker moiety to the N-terminus of the pentapeptide (**12**) produces a PROTAC with a superior degradative ability than one linked at the C-terminus (**16**). **B.** ER degradation by PROTACs with varying linker lengths (9, 12, 16, 19 and 21 atom chain length). **11–13** show the best ER degradation at these concentrations. **C.** Examination of the ER degradation at lower concentrations confirms the trend favoring PROTACs with linker lengths of 16 atoms or less. **D.** A time-dependent ER degradation by selected PROTACs, treated once, as above. **E.** A time-dependent ER degradation by selected PROTACs with a media change including PROTACs after 48 hours (treated twice), demonstrating the superior efficacy of **13**.

Fig. 4.

Degradation of the ER by PROTACs is proteasome-dependent and E3 ligase recognition residue-dependent. **A.** PROTAC-mediated ER degradation is inhibited in a dose-dependent manner by the general proteasome inhibitor, epoxomicin. **B.** The hydroxyproline residue is critical for pVHL recognition of the PROTAC, as a mutant containing norleucine (**17**) is unable to degrade the ER. **C.** Immunofluorescence data supporting the western blotting results of **A** and **B**. Epoxomicin causes the accumulation of the ER (green), while **17** does not cause ER degradation when compared to controls. Blue staining (DAPI) indicates the nucleus.

Fig. 5.

Determination of PROTAC efficacy. **A.** PROTACs having different linker lengths have similar ER binding activity. Note that compounds **12** and **16**, which differ in their attachment to the linker, result in similar ER binding affinity. **B.** Relative cell viability (MTS) results obtained from MCF7 cells after 48 hour exposure to PROTACs or tamoxifen. Compound **13** has an IC_{50} value similar to that of tamoxifen in this cell line and is the PROTAC which show the best efficacy in this assay. **C.** A PROTAC's ability to degrade the ER is correlated with its cytotoxic activity, which varies as a function of linker length. The ER degraded columns show quantification of western blotting results (see Fig. 3B) for ER- as a percentage of vehicle control. The IC_{50} values are from in Fig. 5B. The 16 atom linker (**13**) is superior for our ER-based PROTACs.

Scheme 1.

Synthesis of an estradiol intermediate containing an amine functional group at the C-7 position.

Scheme 2.

Syntheses of PROTACs with varying linker length (**11–15**), **16** in which the E2 domain is attached at the C-terminus of the pentapeptide, and **17**, a negative control PROTAC in which the hydroxyproline is replaced with norleucine. R = -Leu-Ala-Pro^{OH}-Tyr-Ile-OBzl. R* = -Leu-Ala-Nle-Leu-Tyr-Ile-OBzl. R** = Z-Leu-Ala-Pro^{OH}-Tyr-Ile-.