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Chemical Genetic Control of Protein Levels: Selective in Vivo Targeted Degradation

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Abstract: Genetic loss of function analysis is a powerful method for the study of protein function. However, some cell biological questions are difficult to address using traditional genetic strategies often due to the lack of appropriate genetic model systems. Here, we present a general strategy for the design and syntheses of molecules capable of inducing the degradation of selected proteins in vivo via the ubiquitin−proteasome pathway. Western blot and fluorometric analyses indicated the loss of two different targets: green fluorescent protein (GFP) fused with FK506 binding protein (FKBP12) and GFP fused with the androgen receptor (AR), after treatment with PROteolysis TArgeting Chimeric moleculeS (PROTACS) incorporating a FKBP12 ligand and dihydrotestosterone, respectively. These are the first in vivo examples of direct small molecule-induced recruitment of target proteins to the proteasome for degradation upon addition to cultured cells. Moreover, PROTAC-mediated protein degradation offers a general strategy to create "chemical knockouts," thus opening new possibilities for the control of protein function.

Introduction

The selective loss of critical cellular proteins and subsequent analysis of the resulting phenotypes have proven to be extremely useful in genetic studies of in vivo protein function. In recent years, genetically modified knockout cell lines and animals have allowed biological research to advance with unprecedented speed. Chemical genetic approaches, using small molecules to induce changes in cell phenotype, are complementary to traditional genetics. Many chemical genetic strategies use knowledge gained from natural product mode of action studies,[1−3] while others employ chemical inducers of dimerization to manipulate intracellular processes.[4−7] To date, however, there have been few attempts to design small molecules which induce the destruction (rather than inhibition) of a targeted protein in an otherwise healthy cell. Access to such reagents would provide a chemical genetic alternative to the traditional ways of interfering with protein function, resulting in "chemical knockouts". Importantly, a small molecule capable of inducing this process could do so without any genetic manipulation of the organism, thus allowing one to target proteins that are not readily accessible by traditional genetic means (i.e., genes essential for proliferation and early development).

Protein expression can be described as occurring on three levels: DNA, RNA, and post-translation. Consequently, interference with protein function may be approached from each of these levels. Genetic knockouts disrupt protein function at the DNA level by directly inactivating the gene responsible for a protein product. On the RNA level, removal of a protein of interest may be accomplished by RNA interference (RNAi). RNAi causes the degradation of mRNA within the cell, preventing the synthesis of a protein, and often resulting in a "knockdown" or total knockout of protein levels. Interference with gene products at the post-translational level would involve degradation of the protein after it has been completely expressed. To date, interference with proteins on the post-translation level is the least explored.

In principle, targeted proteolytic degradation could be an effective way to accomplish the removal of a desired gene product at the post-translational level. Given the central role of

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\# Department of Pharmacology, Yale University.

(1) Harding, M. W.; Galat, A.; Uehling, D. E.; Schreiber, S. L. *Nature* **1989**, *341*, 758−60.
(2) Sin, N.; Meng, L.; Wang, M. Q. W.; Wen, J. J.; Bornmann W. G.; Crews, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 6099−6103.
(3) Kwok, B. H. B.; Koh, B.; Ndubuisi, M. I.; Elofsson, M.; Crews, C. M. *Chem. Biol.* **2001**, *14*, 1−8.
(4) Spencer, D. M.; Wandless, T. J.; Schreiber, S. L.; Crabtree, G. R. *Science* **1993**, *262*, 1019−1024.
(5) Belshaw, P. J.; Ho, S. N.; Crabtree, G. R.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4604−4607.
(6) Lin, H.; Abdia, W. M.; Sauer, R. T.; Cornish, V. W. *J. Am. Chem. Soc.* **2000**, *122*, 4247−4248.
(7) Lin, H.; Cornish, V. W. *Angew. Chem., Int. Ed.* **2001**, *40*, 871−875.

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the ubiquitin−proteasome pathway in protein degradation within the cell,[8] reagents capable of redirecting the substrate specificity of this pathway would be useful as experimental tools for modulating cellular phenotype and potentially act as drugs for inducing the elimination of disease-promoting proteins. We present here a general strategy for designing molecules capable of inducing the proteolysis of a targeted protein via the ubiquitin−proteasome pathway, as well as the first evidence that such molecules are effective upon addition to living cells.

Protein degradation, like protein synthesis, is an essential part of normal cellular homeostasis. As the major protein degradation pathway, the ATP-dependent ubiquitin−proteasome pathway has been implicated in the regulation of cellular processes as diverse as cell cycle progression,[9] antigen presentation,[10] the inflammatory response,[11] transcription,[12] and signal transduction.[13] The pathway involves two discrete steps: (i) the specific tagging of the protein to be degraded with a polyubiquitin chain and (ii) the subsequent degradation of the tagged substrate by the 26S proteasome, a multicatalytic protease complex. Ubiquitin, a highly conserved 76 amino acid protein,[14] is conjugated to the target protein by a three-part process. First, the C-terminal carboxyl group of ubiquitin is activated by a ubiquitin-activating enzyme (E1). The thioester formed by attachment of ubiquitin to the E1 enzyme is then transferred via a transacylation reaction to an ubiquitin-conjugating enzyme (E2). Finally, ubiquitin is transferred to a lysine (or, less commonly, the amino terminus) of the protein substrate that is specifically bound by an ubiquitin ligase (E3).[15] Successive conjugation of ubiquitin to internal lysines of previously added ubiquitin molecules leads to the formation of polyubiquitin chains.[16] The resulting polyubiquitinated target protein is then recognized by the 26S proteasome, whereupon ubiquitin is cleaved off and the substrate protein threaded into the proteolytic chamber of the proteasome. Importantly, substrate specificity of the ubiquitin−proteasome pathway is conferred by the E3 ligases. Each E3 ligase or recognition subunit of a multiprotein E3 ligase complex binds specifically to a limited number of protein targets sharing a particular destruction sequence. The destruction sequence may require chemical or conformational modification (e.g., phosphorylation) for recognition by E3 enzymes.[17,18]

Recently, we demonstrated a strategy for inducing the ubiquitination and ensuing proteolytic degradation of a targeted protein in vitro. This approach uses heterobifunctional molecules known as PROteolysis TArgeting Chimeric moleculeS (PROTACS), which comprise a ligand for the target protein, a linker moiety, and a ligand for an E3 ubiquitin ligase.[19] In that proof of principle experiment the degradation of a stable protein, methionine aminopeptidase 2 (MetAP-2), was induced in a

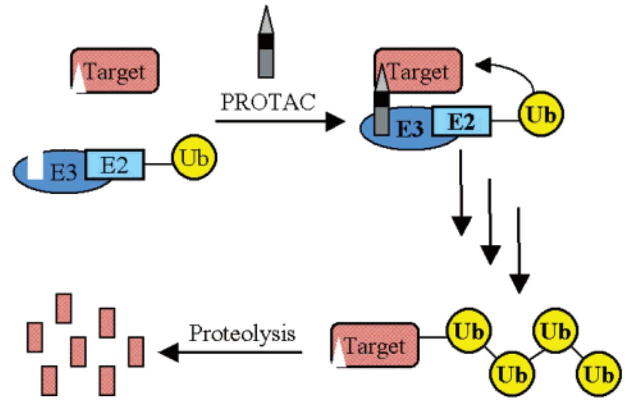


Figure 1. Targeted proteolysis using a PROTAC molecule. Ub = ubiquitin, target = target protein, E3 = E3 ubiquitin ligase complex, and E2 = E2 ubiquitin transfer enzyme.

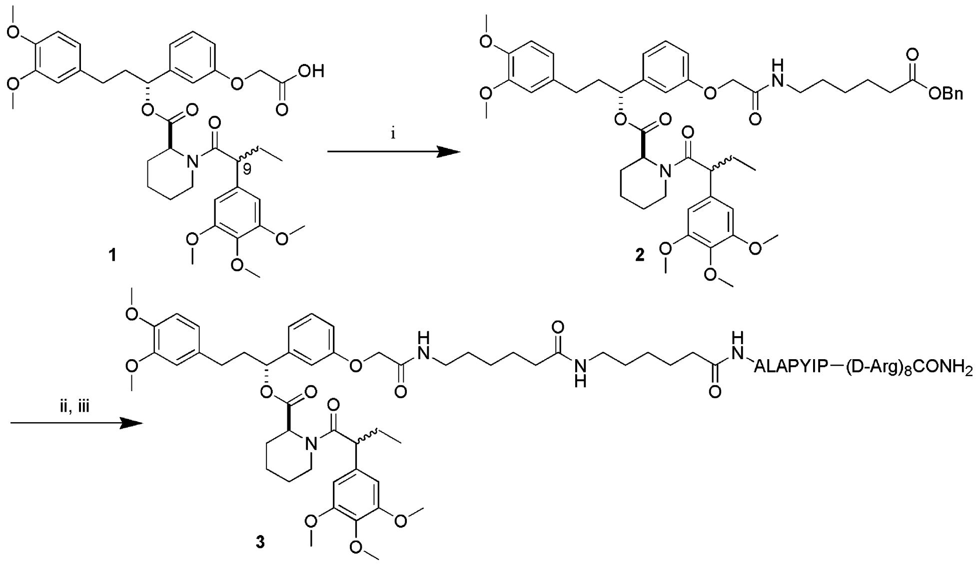
cellular lysate upon the addition of a PROTAC (referred to as PROTAC-1) consisting of the known MetAP-2 ligand, ovalicin, joined to a peptide ligand for the ubiquitin ligase complex SCF$^{\beta\mathrm{TrCP}}$. By bridging MetAP-2 and an E3 ligase, PROTAC-1 initiated the ubiquitination and proteasome-mediated degradation of MetAP-2 (Figure 1). We have also recently shown that an estradiol-based PROTAC (PROTAC-2) could promote the ubiquitination of the human estrogen receptor (hERα) in vitro. Furthermore, a dihydrotestosterone (DHT)-based PROTAC (PROTAC-3), when microinjected into cells, was capable of inducing the degradation of the androgen receptor.[20] Encouraged by our success with PROTACS-1, −2, and −3, we next directed our efforts toward the design of molecules capable of inducing proteolysis simply upon addition to cells. Additionally, the design of new PROTACS takes into account the desire to minimize the amount of molecular biological manipulations necessary to effect degradation to perturb the system as little as possible outside the desired degradation.

Results

Development of a Cell Permeable PROTAC: PROTAC-4. For the design of PROTAC-4, we used a protein target/ligand pair developed by ARIAD Pharmaceuticals. The F36V mutation of FK506 binding protein (FKBP12) generates a "hole" into which the artificial ligand AP21998 (**1**) fits via a hydrophobic "bump," thus conferring specificity of this particular ligand to the mutant FKBP over the wild-type protein.[21,22] Inclusion of AP21998 as one domain of PROTAC-4 thus allows it to target (F36V)FKBP12 proteins orthogonally, without disrupting endogenous FKBP12 function. Given the lack of small-molecule E3 ubiquitin ligase ligands, the seven amino acid sequence ALAPYIP was chosen for the E3 recognition domain. This sequence has been shown to be the minimum recognition domain for the von Hippel−Lindau tumor suppressor protein (VHL),[23] part of the VBC−Cul2 E3 ubiquitin ligase complex. Under normoxic conditions, a proline hydroxylase catalyzes the hydroxylation of hypoxia inducible factor 1α (HIF1α) at P564[24]

(8) Myung, J.; Kim, K.; Crews, C. M. *Med. Res. Rev.* **2001**, *21*, 245−273.
(9) Koepp, D. M.; Harper, J. W.; Elledge, S. J. *Cell* **1999**, *97*, 431−434.
(10) Rock, K. L.; Goldberg, A. L. *Annu. Rev. Immunol.* **1995**, *17*, 739−779.
(11) Ben-Neriah, Y. *Nat. Immunol.* **2002**, *3*, 20−26.
(12) Muratani, M.; Tansey, W. P. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 192−201.
(13) Hershko, A.; Ciechanover, A. *Annu. Rev. Biochem.* **1998**, *67*, 425−479.
(14) Vijay-Kumar, S.; Bugg, C. E.; Wilkinson, K. D.; Vierstra, R. D.; Hatfield, P. M.; Cook, W. J. *J. Biol. Chem.* **1987**, *262*, 6396−6399.
(15) Breitschopf, K.; Bengal, E.; Ziv, T.; Admon, A.; Ciechanover, A. *EMBO J.* **1998**, *17*, 5964−5973.
(16) Pickart, C. M. *Annu. Rev. Biochem.* **2001**, *3*, 503−533.
(17) Yaron, A.; Hatzubal, A.; Davis, M.; Lavon, I.; Amit, S.; Manning, A. M.; Andersen, J. S.; Mann, M.; Mercurio, F.; Ben-Neriah, Y. *Nature* **1998**, *396*, 590−594.
(18) Crews, C. M. *Curr. Opin. Chem. Biol.* **2003**, *7*, 534−539.
(19) Sakamoto, K. M.; Kim, K. B.; Kumagai, A.; Mercurio, F.; Crews, C. M.; Deshaies, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 8554−8559.
(20) Sakamoto, K.; Kim, K. B.; Verma, R.; Rasnick, A.; Stein, B.; Crews, C. M.; Deshaies, R. J. *Mol. Cell. Proteomics* **2003**, *2*, 1350−1358.
(21) Yang, W.; Roxamus, L. W.; Narula, S.; Rollins, C. T.; Yuan, R.; Andrade, L. J.; Ram, M. K.; Phillips, T. B.; van Schravendijk, M. R.; Dalgarno, D.; Clackson, T.; Holt, D. *J. Med. Chem.* **2000**, *43*, 1135−1142.
(22) Rollins, C. T.; Rivera, V. M.; Woolfson, D. N.; Keenan, T.; Hatada, M.; Adams, S. E.; Andrade, L. J.; Yaeger, D.; van Schravendijk, M. R.; Holt, D. A.; Gilman, M.; Clackson, T. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7096−7101.

Scheme 1. Synthesis of the AP21998/HIF1 α-Based PROTAC[a]



[a] (i) $H_2N(CH_2)_5CO_2Bn$, EDCI, DMAP. (ii) H_2, Pd/C. (iii) $H_2N(CH_2)_5CONH$-ALAPYIP-(D-Arg)$_8$-NH_2, PyBrOP, DIPEA, DMF.

(the central proline in the ALAPYIP sequence), resulting in recognition and polyubiquitination by VHL. HIF1α is thus constitutively ubiquitinated and degraded under normoxic conditions.[25,26] Finally, a poly-D-arginine tag was included on the carboxy terminus of the peptide sequence to confer cell permeability and resist nonspecific proteolysis. Polyarginine sequences fused to proteins have been shown to facilitate translocation into cells[27,28] via a mechanism that mimics that of the Antennapedia[29] and HIV Tat proteins.[30] Because a molecule fused to the polyarginine sequence should in principle be cell permeable, the necessity of PROTAC microinjection is circumvented. This design element also allows greater flexibility in the types of ligands that could be used in future PROTACs, since polarity of the compound is no longer an issue for membrane permeability. It was hypothesized that PROTAC-4 would enter the cell, be recognized and hydroxylated by a prolyl hydroxylase, and subsequently be bound by both the VHL E3 ligase and the mutant FKBP12 target protein. PROTAC-mediated recruitment of FKBP12 to the VBC−Cul2 E3 ligase complex would be predicted to induce FKBP12 ubiquitination and degradation as in Figure 1.

The F36V FKBP12 ligand AP21998 (**1**) was synthesized as previously described,[21,22] as an approximately 1:1 mixture of diastereomers at C9. Treatment of **1** with the benzyl ester of aminocaproic acid followed by removal of the benzyl group afforded **2** in 85% crude yield after two steps. It is important to note that although this material was carried through as a mixture of two diastereomers at C9, each diastereomer has previously been shown to bind to the target.[22] Standard peptide coupling conditions were used to label the peptide sequence. HPLC purification yielded **3** (PROTAC-4) with 17% recovery from **1** (Scheme 1).

To monitor the abundance of the targeted protein, a vector capable of expressing the mutant FKBP12 fused to enhanced green fluorescent protein (EGFP) was generated. In this way, proteolysis of FKBP12 could be monitored by loss of intracellular fluorescence. This vector was then used to generate a HeLa cell line stably expressing the EGFP−(F36V)FKBP12. Bright field and fluorescent photographs of the cells were taken before and 2.5 h after treatment with PROTAC-4 (**3**). As shown in Figure 2A−D, EGFP−FKBP12 was retained in those cells treated with DMSO, but lost in cells treated with 25 μM PROTAC-4 for 2.5 h. Western blot analysis of cells treated with PROTAC-4 also indicated loss of EGFP−FKBP12 relative to an equal number of cells treated with DMSO (Figure 2I). As a control, cells were treated with uncoupled **1** and the HIF-polyarginine peptide fragment (Figure 2E,F). These cells retained fluorescence, indicating that the two domains require a chemical bond to each other to exert a biological effect. To investigate whether VHL was required for PROTAC-4-mediated EGFP−FKBP12 degradation, the renal carcinoma cell line 786-O[31] was used. 786-O cells failed to produce VHL protein and thus lack a functional VBC−Cul2 E3 ligase complex. 786-O cells stably expressing the degradation substrate EGFP−FKBP12 retained fluorescence despite treatment with 25 μM PROTAC-4 for 2.5 h (Figure 2G,H), confirming that the E3 ligase is required for PROTAC-4 activity. Finally, similar cell density and morphology in bright field images before (Figure 2I) and after (Figure 2J) treatment with 25 μM PROTAC-4 for

(23) Hon, W.; Wilson, M. I.; Harlos, K.; Claridge, T. D. W.; Schofield, C. J.; Pugh, C. W.; Mazwell, P. H.; Ratcliffe, P. J.; Stuart, D. I.; Jones, E. Y. *Nature* **2002**, *417*, 975−978.
(24) Epstein, A. C.; Gleadle, J. M.; McNeill, L. A.; Heritson, K. S.; O'Rourke, J.; Mole, D. R.; Mukherji, M.; Metzen, E.; Wilson, M. I.; Dhanda, A.; Tian, Y. M.; Masson, M.; Hamilton, D. L.; Jaakkola, P.; Barstead, R.; Hodgkin, J.; Mazwell, P. H.; Pugh, C. W.; Schofield, C. J.; Ratcliffe, P. J. *Cell* **2001**, *107*, 43−54.
(25) Ohh, M.; Park, C. W.; Ivan, M.; Hoffmann, M. A.; Kim, T. Y.; Huang, L. E.; Pavletich, N.; Chau, V.; Kaelin, W. G. *Nat. Cell Biol.* **2000**, *2*, 423−427.
(26) Tanimoto, K.; Makino, Y.; Pereira, T.; Poellinger, L. *EMBO J.* **2000**, *19*, 4298−4309.
(27) Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003−13008.
(28) Kirschberg, T. A.; VanDeusen, C. L.; Rothbard, J. B.; Yang, M.; Wender, P. A. *Org. Lett.* **2003**, *5*, 3459−3462.
(29) Derossi, D.; Joliot, A. H.; Chassaing, G.; Prochiants, A. *J. Biol. Chem.* **1994**, *269*, 10444−10450.
(30) Fawell, S.; Seery, J.; Daikh, Y.; Moore, C.; Chen, L. L.; Pepinsky, B.; Barsoum, J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 664−668.
(31) Baba, M.; Hirai, S.; Yamada-Okabe, H.; Hamada, K.; Tabuchi, H.; Kobayashi, K.; Kondo, K.; Yoshida, M.; Yamashita, A.; Kishada, T.; Nakaigawa, N.; Nagashima, Y.; Kubota, Y.; Yao, M.; Ohno, S. *Oncogene* **2003**, *22*, 2728−2738.

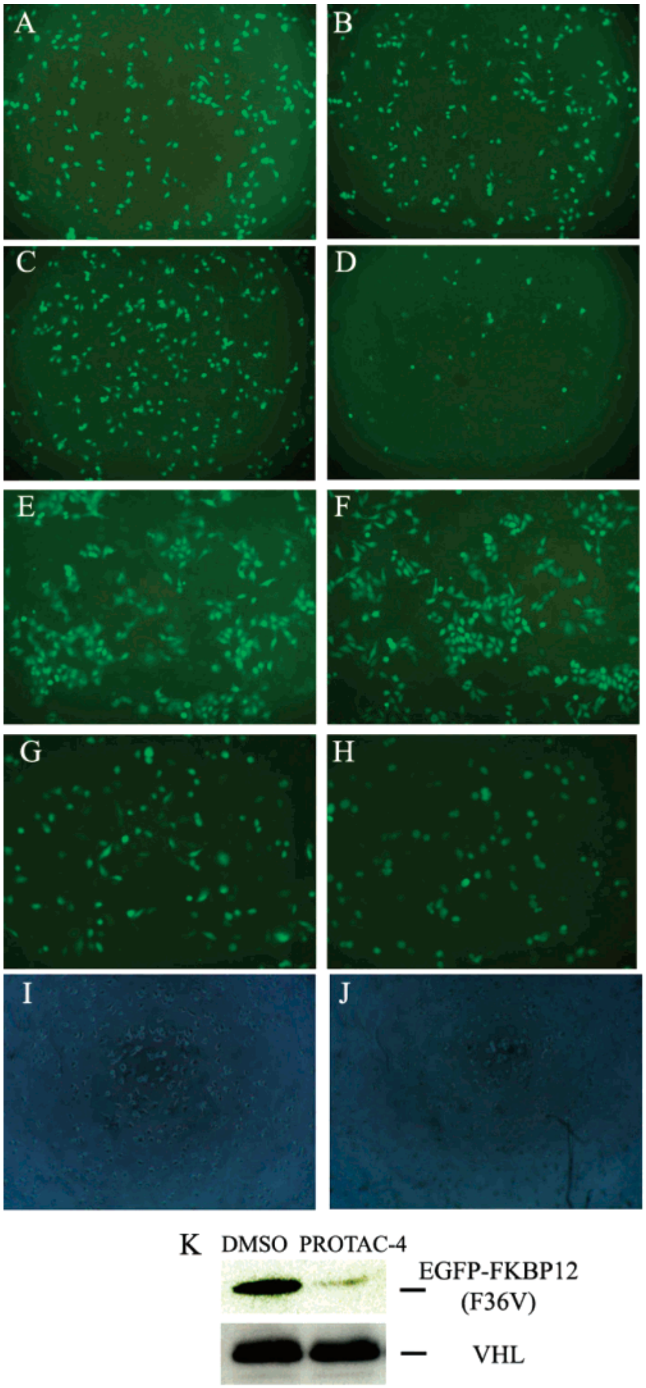
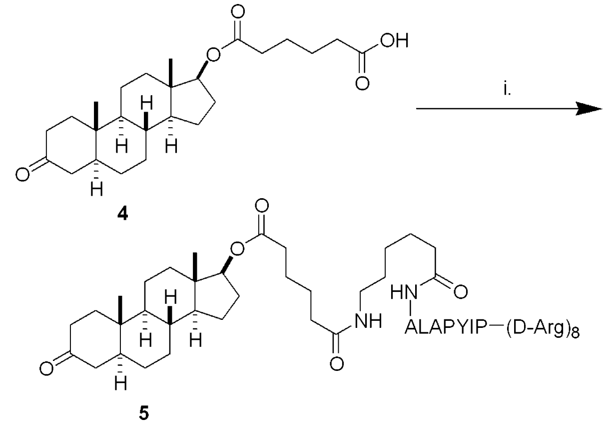


Figure 2. PROTAC-4 (**3**) mediates EGFP−FKBP degradation in a VHL-dependent manner. No change in fluorescence is observed before (A) and 2.5 h after (B) treatment in DMSO control, while a significant change is observed between before (C) and 2.5 h after (D) treatment with 25 μM **3**. Cells treated with 25 μM **1** and 25 μM HIF-(D-Arg)$_8$ peptide show no difference before (E) and 2.5 h after (F) treatment. 786-O$^{EGFP-FKBP}$ cells do not lose fluorescence before (G) or 2.5 h after (H) treatment with 25 μM **3**. Bright field images of cells before (I) and 2.5 h after (J) treatment with 25 μM **1** affirm constant cell density and morphology. Western blot analysis (K) with monoclonal anti-GFP antibodies confirms loss of EGFP−FKBP in cells treated with 25 μM **3** (PROTAC-4) for 2.5 h compared to an equal load from vehicle (DMSO) treated cells.

2.5 h confirm that cells are capable of surviving treatment with a PROTAC molecule.

Implementation of a DHT-Based PROTAC: PROTAC-5. To test the robustness of this approach for the induction of intracellular protein degradation, we next used a well understood protein−ligand pair which occurs in nature. The testosterone/androgen receptor pair was particularly attractive because it has been shown that the androgen receptor (AR) can promote the growth of prostate tumor cells, even in some androgen-independent cell lines.[32] In those same cell lines, it has been shown that inhibition of AR represses growth.[32] We hypothesized that a PROTAC could be utilized to degrade AR, potentially yielding a novel strategy to repress tumor growth. With this in mind, the design of PROTAC-5, **5**, contains DHT as the ligand for AR as well as the HIF-polyarginine peptide sequence which was successful with PROTAC-4. Known DHT derivative **4**[33] was successfully coupled to the HIF-polyarginine peptide with standard peptide coupling conditions (Scheme 2). To monitor protein degradation by fluorescence analysis, HEK293 cells stably expressing GFP-AR (293^{GFP-AR}) were treated with increasing concentrations of PROTAC-5. Within 1 h, a significant decrease in GFP-AR signal was observed in cells treated with 100, 50, and 25 μM PROTAC-5, but not in the DMSO control (Figure 3, parts A−F, I, L). Western blot analysis with anti-AR antisera verified the downregulation of GFP-AR in cells treated with 25 μM PROTAC-5 compared to DMSO control or nontreated cells (Figure 3M). PROTAC-5 concentrations lower than 25 μM did not result in GFP-AR degradation (data not shown). Pretreatment of cells with epoxomicin, a specific proteasome inhibitor,[34] prevented degradation of GFP-AR (Figure 3, part H: light field, K: fluorescent), indicating that the observed degradation was proteasome-dependent. This result was also verified by Western blot (Figure 3N). It should be noted that decreased cell density in the epoxomicin experiments are most likely due to the inherent toxicity of epoxomicin itself, rather than from a toxic effect of the PROTAC. This is supported by the viability of cells treated with PROTAC-5, as seen in Figure 3B,C.

Scheme 2. Synthesis of a DHT/HIF1 α-Based PROTAC (PROTAC-3)[a]



[a] (i) $H_2N(CH_2)_5$CONH-ALAPYIP-(D-Arg)$_8$-NH_2, EDCI, DMAP, DMF.

Competition experiments with testosterone also inhibited PROTAC-5 from inducing GFP-AR degradation (Figure 4 A-D). In addition, cells treated only with testosterone retained all fluorescence, as did cells treated with the HIF-polyarginine peptide (Figure 4G,H). Finally, cells treated with both testoster-

(32) Debes, J. D.; Schmidt, L. J.; Huang, H.; Tindall, D. J. *Cancer Res.* **2002**, *62*, 5632−5636.
(33) Stobaugh, M. E.; Blickenstaff, R. *Steroids* **1990**, *55*, 259−262.
(34) Meng, L.; Mohan, R.; Kwok, B. H. K.; Elofsson, M.; Sin, N.; Crews, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10403−10408.

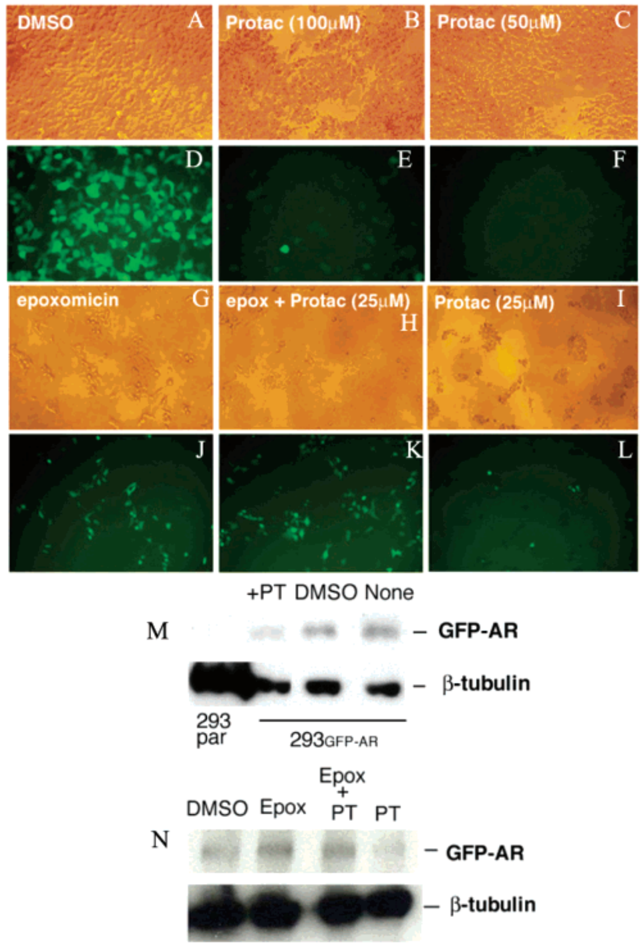


Figure 3. DHT-HIF PROTAC-5 (**5**) mediates GFP-AR degradation in a proteasome-dependent manner. One hour after treatment, 293^{GFP-AR} cells treated with a 100 μM (B light field, E fluorescent) or 50 μM (C light field, F fluorescent) concentration of **5** lose fluorescence, while the DMSO control (A light field, D fluorescent) retains fluorescence. Cells treated with 10 μM epoxomicin (G light field, J fluorescent) and pretreated with 10 μM epoxomicin for 4 h followed by treatment with 25 μM **5** for 1 h (H light field, K fluorescent) retain fluorescence, while cells treated only with 25 μM **5** lose fluorescence after 1 h (I light field, L fluorescent). Western blot analysis confirms loss of GFP-AR after treatment with PROTAC **5** (+PT) relative to a loading control (M), while inhibition of the proteasome with epoxomicin (Epox) inhibits degradation (N).

one and the HIF-polyarginine peptide together also retained fluorescence, indicating again that both domains needed to be chemically linked to observe degradation (Figure 4F). It is important to note again that the cells survived treatment with PROTAC-5, indicating that the strategy of utilizing the ubiquitin−proteasome pathway for targeted degradation does not necessarily cause a toxic effect.

Discussion

These experiments highlight the general applicability of a novel strategy to target and degrade proteins in vivo. Although this technique has been shown to be effective previously in vitro, this is the first example of synthesized molecules which are capable of inducing the degradation of a targeted protein upon addition to cells. Use of a GFP fusion protein provided a convenient method to monitor PROTAC-induced degradation, but is not inherently necessary to the design of the molecule. In principle, no molecular biological manipulations are needed to implement a PROTAC molecule. This technique therefore

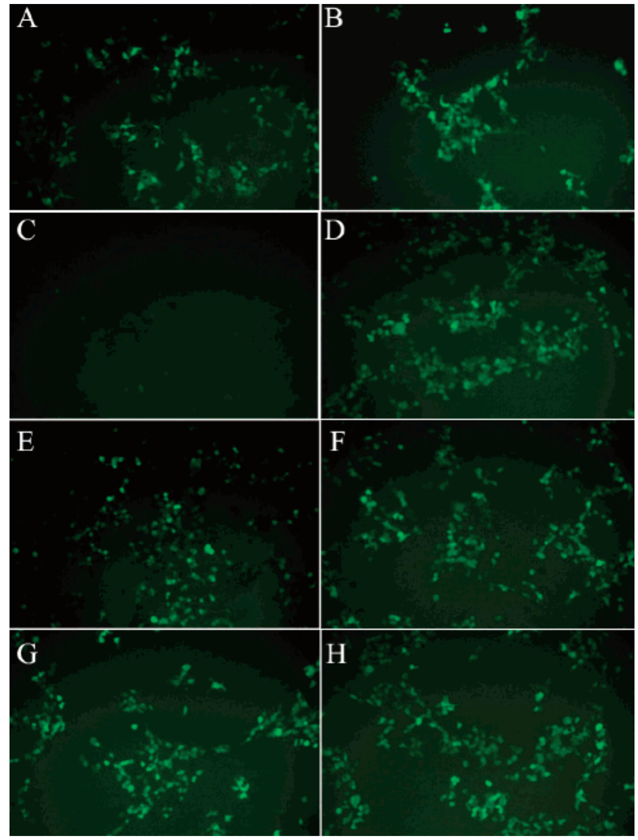


Figure 4. A chemical bond between the HIF-(D-Arg)$_8$ peptide and DHT is required for PROTAC-5-induced degradation of GFP-AR. Cells were treated with (A) no treatment, (B) DMSO (equal volume), (C) 25 μM PROTAC-3, (D) 25 μM PROTAC-5 + 10-fold molar excess testosterone, (E) 25 μM PROTAC-5 + 10-fold molar excess (250 μM) HIF-D-Arg peptide, (F) 25 μM HIF-D-Arg peptide + 25 μM testosterone added separately, (G) 25 μM DHT, and (H) 25 μM HIF-D-Arg peptide.

provides a novel approach to the study of protein function without genetically modifying the host cell. Moreover, the modularity of the PROTAC design offers the possibility to synthesize similar PROTAC molecules targeting a variety of intracellular targets. These experiments have shown that the ligand for the target protein can be varied using both natural and synthetic ligands to degrade effectively targeted GFP fusion proteins. Although the linker length has not been fully explored, a spacer consisting of two aminocaproic acids (12 atoms) has been shown to be flexible enough to accommodate some structural variation in the target and E3 ligase proteins yet remain functional. Since ubiquitination occurs most commonly on an exposed lysine, different spacer lengths may be required to accommodate the structures of different target proteins.

Small molecules have previously been implicated in inducing ubiquitination and degradation of proteins; most notably geldanamycin derivatives act by controlling target interaction with molecular chaperones.[35−38] However, there are often specificity issues with these approaches, and the exact mechanism of

(35) Kuduk, S. D.; Zheng, F. F.; Sepp-Lorenzino, L.; Rosen, N.; Danishefsky, S. J. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1233−1238.
(36) Kuduk, S. D.; Harris, C. R.; Zheng, F. F.; Sepp-Lorenzino, L.; Ouerfelli, Q.; Rosen, N.; Danishefsky, S. J. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1303−1306.
(37) Zheng, F. F.; Kuduk, S. D.; Chiosis, G.; Münster, P. N.; Sepp-Lorenzino, L.; Danishefsky, S. J.; Rosen, N. *Cancer Res.* **2000**, *60*, 2090−2094.
(38) Citri, A.; Alroy, I.; Lavi, S.; Rubin, C.; Xu, W.; Grammatikakis, N.; Patterson, C.; Neckers, L.; Fry, D. W.; Yarden, Y. *EMBO J.* **2002**, 2407−2417.

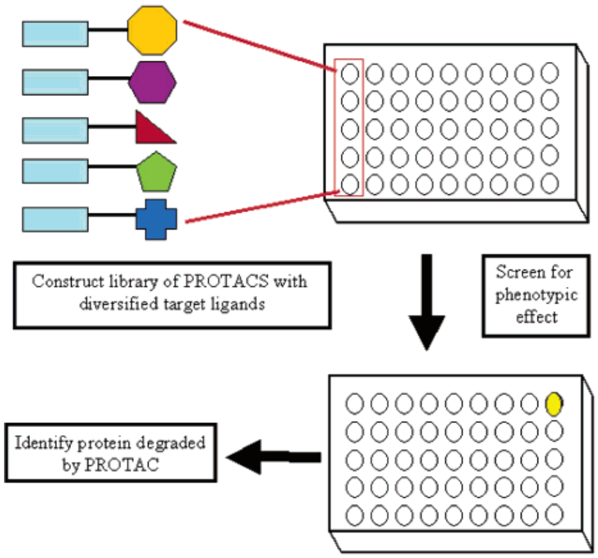


Figure 5. Potential use of PROTACS in a chemical genetic screen.

induced degradation is not clear. Interference with gene products at the post-translational level has also been successfully demonstrated by Howley and co-workers,[39] who used known protein−protein interacting domains. Their approach, while successful, required significant manipulation of the cell lines in question to observe an effect. Both of these methods are significantly less direct and flexible than PROTACS. In addition, the PROTAC strategy represents the first attempt to develop a general method for small molecule-induced targeted proteolysis via the ubiquitin−proteasome pathway in intact cells.

PROTACS could in principle be used to target almost any protein within a cell and selectively initiate its degradation, resulting in a "chemical knockout" of protein function. A notable advantage to this strategy is that proteolysis is not dependent on the active-site inhibition of the target; any unique site of a protein may be targeted, provided that there are exposed lysines within proximity for the attachment of ubiquitin. Because some E3 ligases are expressed in a tissue-specific manner, this also raises the possibility that PROTACS could be used as tissue-specific drugs.

Several other applications for this technology can be envisioned. First, PROTACS could be used to control a desired cellular phenotype, for example, via the induced degradation of a crucial regulatory transcription factor which is difficult to target pharmaceutically. "Chemical knockout" of a protein could prove viable as an alternative for a genetic knockout, which would be extremely valuable in the study of protein function. This strategy could also provide significantly more temporal or dosing control than gene inactivation at the DNA or RNA level. Second, libraries of PROTACS could be used to screen for phenotypic effects in a chemical genetic fashion. This strategy could be used either to identify novel ligands for a target or to identify new therapeutically vulnerable protein targets by studying phenotypic change as a result of selective protein degradation (Figure 5). This chemical genetic strategy would employ a library of PROTAC molecules with identical E3 ubiquitin ligase domains but chemically diverse target ligands. After PROTAC library incubation with cultured cells and detection of the desired cellular phenotype (e.g., inhibition of pro-inflammatory signaling), one could identify the protein that was degraded by incubation with the PROTAC. A number of approaches could be used to identify the PROTAC-targeted protein, including affinity chromatography and differential proteomic technologies such as ICAT.[40] In a modification of this strategy, a library of PROTACS could be screened to identify a ligand for a particular target by monitoring degradation of the target protein (e.g., loss of GFP fusion protein). Finally, PROTACS could be used as drugs to remove toxic or disease-causing proteins. This strategy is particularly appealing since many diseases, including several cancers, are dependent on the presence or overexpression of a small number of proteins. The large number of potential uses for this technology, coupled with the success of these experiments, suggests that PROTACS could find broad use in the fields of cell biology, biochemistry, and potentially medicine.

Experimental Section

A. Materials. (F36V)FKBP12 expression vector was generously provided by ARIAD Pharmaceuticals (Cambridge, MA), and GFP-AR expression plasmid was a gift from Dr. Charles Sawyers (HHMI, UCLA). Epoxomicin[41] and AP21998[21,22] were synthesized as previously described. Dihydrotestosterone and testosterone were obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal antibody recognizing VHL was purchased from Oncogene (San Diego, CA), antibodies recognizing GFP and β-tubulin were obtained from Santa Cruz Biotech (Santa Cruz, CA), and polyclonal antibody against the androgen receptor was from United Biomedical, Inc. (Hauppauge, NY). HEK293, 786-O, and HeLa cells were purchased from the American Type Culture Collection (Manassas, VA). Tissue culture medium and reagents were obtained from GIBCO-Invitrogen (Carlsbad, CA).

B. Tissue Culture. HeLa cells, 786-O cells, and HEK 293 cells were separately cultured in D-MEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine. All cell lines were maintained at a temperature of 37 °C in a humidified atmosphere of 5% CO_2. To generate cells stably expressing a particular fluorescent target protein, the parent cell line was grown to 70% confluency and transfected using calcium phosphate precipitation of the designated cDNA. Following transfection, cells were split 1:10 into culture medium supplemented with 600 μg/mL G418 (GIBCO-Invitrogen). Individual clones which optimally expressed fluorescent target protein were identified and expanded under selection for further experimentation.

C. Detection of PROTAC-Induced Degradation by Fluorescence Microscopy. Cells stably expressing fluorescent target protein were plated into 96 well plates (HeLa$^{EGFP-FKBP}$ cells plated at 4000 cells/well and HEK293^{GFP-AR} cells plated at 60 000−100 000 cells/well). Synthesized PROTACS were dissolved in DMSO vehicle at a final concentration of 1%. Disappearance of target protein in vivo was monitored by fluorescence microscopy at an excitation wavelength of 488 nm.

D. Detection of PROTAC-Induced Degradation by Western Blot. Whole cell lysates were prepared from HeLaEGFP−FKBP cells treated with PROTAC-4 and with HEK293GFP-AR cells treated with PRTOAC-5 by lysing the cells in hot Laemmli buffer. Lysates were subjected to 8% polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membrane. Membranes were blocked in 3% nonfat milk in TBS supplemented with 0.1% Triton X-100 and 0.02% sodium azide. Lysates from HeLa$^{EGFP-FKBP}$ cells treated with

(39) Zhou, P.; Howley, P. *Mol. Cell.* **2000**, *6*, 751−756.

(40) Han D. K.; Eng J.; Zhou, H.; Aebersold, R. *Nat Biotechnol.* **2001**, *19*, 946−951.

(41) Sin, N.; Kim, K. B.; Elofsson, M.; Meng, L.; Auth, H.; Kwok, B. H. B.; Crews, C. M. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2283−2288.

PROTAC-4 were probed with anti-GFP (1:1000) and anti-VHL (1:1000) antibodies, and HEK293GFP-AR cells treated with PROTAC-5 were probed with anti-androgen receptor (1:1000) and anti-β-tubulin (1:200) antibodies. Blots were developed using chemiluminescent detection.

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Supporting Information Available: Preparation and characterization information for compounds **3** and **5** and the HIF-polyarginine peptide (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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