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Article

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Development of a small hybrid molecule that mediates degradation of His-tag fused proteins

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KEYWORDS: His-tag fused protein; protein knockdown; ubiquitin-proteasome system; CRABP-II, Smad2

ABSTRACT. In recent years, the induction of target-protein degradation via the ubiquitin-proteasome system (UPS) mediated by small molecules has attracted attention, and this approach has applications in pharmaceutical development. However, this technique requires a ligand for the target protein that can be incorporated into tailor-made molecules, and there are many proteins for which such ligands have not been found. In this study, we developed a protein-knockdown method that recognizes a His-tag fused to a protein of interest. This strategy theoretically allows comprehensive targeting of proteins of interest by a particular molecule recognizing the tag. As expected, our hybrid molecule **10** [**SNIPER(CH6)**] efficiently degraded His-tagged CRABP-II and Smad2 in cells. This system provides an easy method to determine the susceptibility of proteins of interest to UPS-mediated degradation. Furthermore, we hope that this method will become an efficient tool to analyze the function of the UPS.

Introduction

Understanding intracellular protein functions is crucial for elucidating living systems and disease etiologies, and controlling the expression of proteins is useful in many aspects of biology. In addition, the ability to control specific protein levels within a living organism using a simple technique would be a useful strategy for pharmaceutical development. For these reasons, a variety of techniques for controlling protein expression have been reported,[1-4] In particular, a protein-knockdown strategy for inducing the degradation of proteins of interest (POI) using the ubiquitin-proteasome system (UPS) has attracted attention. Small molecules called PROTACs (proteolysis-targeting chimeras)[5-15] and SNIPERs (specific and non-genetic inhibitors of apoptosis protein [IAP]-dependent protein erasers)[16-21] have been developed for use in this method. These molecules are composed of two different ligands connected by a linker; one is a ligand for the POI, and the other is a ligand for E3 ubiquitin ligase. Thus, these molecules are capable of crosslinking POI and E3 ubiquitin ligase in cells, resulting in the ubiquitylation and subsequent degradation of POI via the UPS (Figure 1a). The main advantages of this technique are that small molecules are chemically stable and the expression of the target protein can be controlled. However, in this method a ligand for each protein of interest is necessary to design and synthesize tailor-made molecules, and therefore, it is difficult to comprehensively analyze which POI are most susceptible to protein knockdown by the UPS.

To solve this problem, degradation inducers for Halo-tag proteins have recently been developed.[22,23] It was found that hybrid small molecules containing a Halo-tag and an E3 ligand were able to bridge between Halo-tag and E3 ligase and facilitate the degradation of Halo-tagged POI (Figure 1b). However, there are some concerns when a high molecular mass tag (33 KDa) is

employed. The large tag might obstruct the function of the fused protein and Lys residues in the tag might be ubiquitylated by degradation-inducer molecules, such as SNIPER, resulting in subsequent degradation of the POI. Other tags, including the His tag[24] and dC10α tag[25] are also used for the imaging of intracellular proteins. The His tag generally consists of six or more His residues attached at the *N*- or *C*-terminus of POI, and a modified His tag consisting of hexa-His and a Cys residue (CH6-tag) has also been reported.[26] The CH6-tag is small and does not contain any Lys residues and the thiol group of the Cys residue is capable of covalently binding to some acceptors such as maleimide and chloroacetyl groups. Therefore, we speculated that the CH6-tag might solve the above-mentioned problems found with using a high molecular mass tag (Table 1). Herein, we describe the development of a SNIPER that bridges between CH6-tag and E3 ligase for the knockdown of CH6-tagged POI via the UPS.

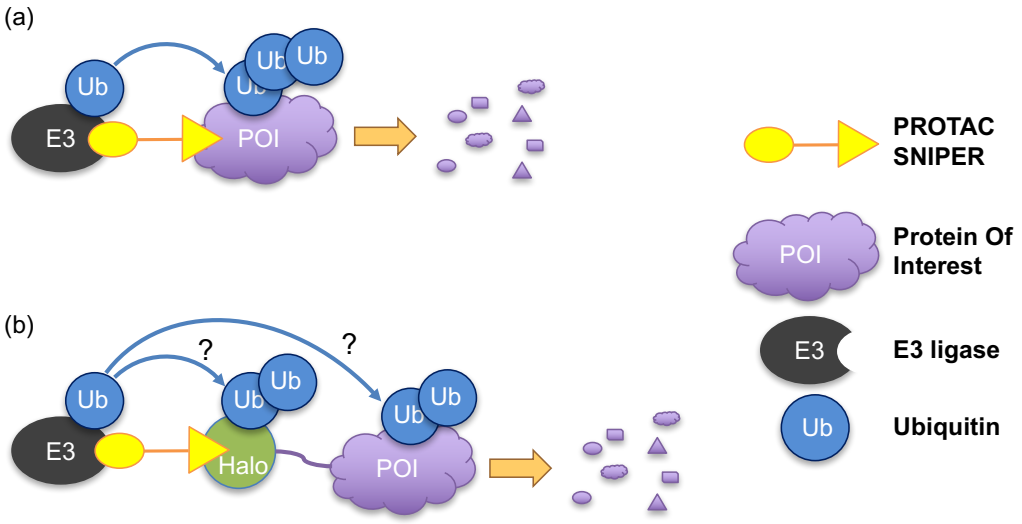


Figure 1. Targeted protein knockdown by hybrid small molecules. (a) Hybrid small molecules, such as SNIPER and PROTAC consisting of a ligand for POI and an E3 ligand, bridge between POI and E3 ligase, and subsequently induce ubiquitylation of POI resulting in proteasomal

degradation of POI. (b) A hybrid molecule targeting Halo-tag fused to POI mediates the degradation of POI. However, this system cannot precisely elucidate the biological implications of the ubiquitylation of POI because it is unclear whether Lys residues in the POI, and not those in the tag portion, are consistently ubiquitylated by the hybrid molecules.

Table 1. Comparison of the present system and published approaches.

	PROTACs/SNIPERs	Halo-tag method	CH6-tag method
Comprehensive application for proteins	Not applicable	Applicable	Applicable
Target endogenous proteins	Applicable	Not applicable	Not applicable
Necessity of gene transfection	Not necessary	Necessary	Necessary
Tag size	-	Large	Small
Ubiquitination of tags	-	Concerned	Not concerned

Results and discussion

The mechanism proposed for the degradation of CH6-tagged POI is shown in Figure 2. Two types of molecules are essential for this technique. One is a degradation inducer **10** [**SNIPER(CH6)**][14] containing nickel nitrilotriacetic acid (Ni-NTA; a His-tag ligand), a maleimide moiety conjugated to a Cys residue in a CH6-tag, and an E3 ligand. However, the degradation inducer itself is not able to penetrate into cells because of the high polarity of Ni-NTA molecules. Therefore, a carrier peptide conjugated with a His-tag is also required to deliver the degradation inducer into cells. These two molecules contribute to the degradation of CH6-tagged POI in the

following manner: 1) The degradation inducer forms a complex with the carrier peptide and then the complex is taken into the target cells. 2) In the cells, the degradation inducer dissociates from the carrier peptide and moves towards CH6-tagged POI, subsequently the maleimide moiety conjugates with the Cys residue in the CH6-tag. 3) Finally, E3 ligase is recruited to the E3 ligand, and POI are ubiquitylated and degraded by the UPS.

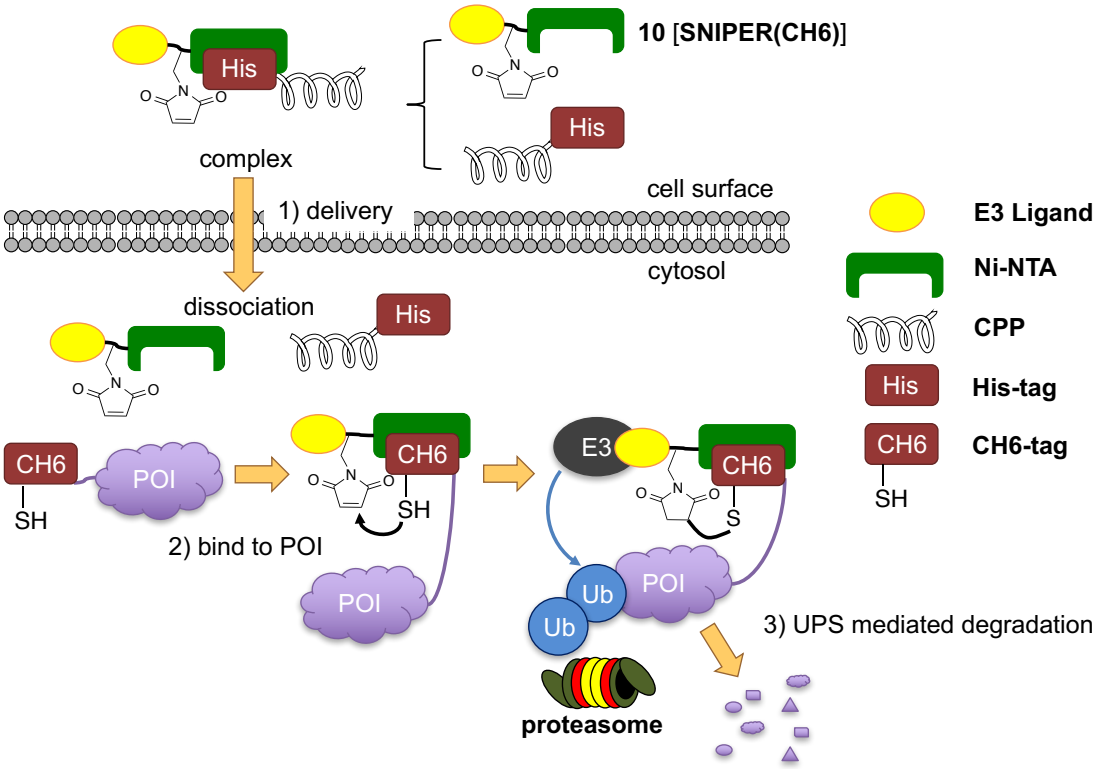
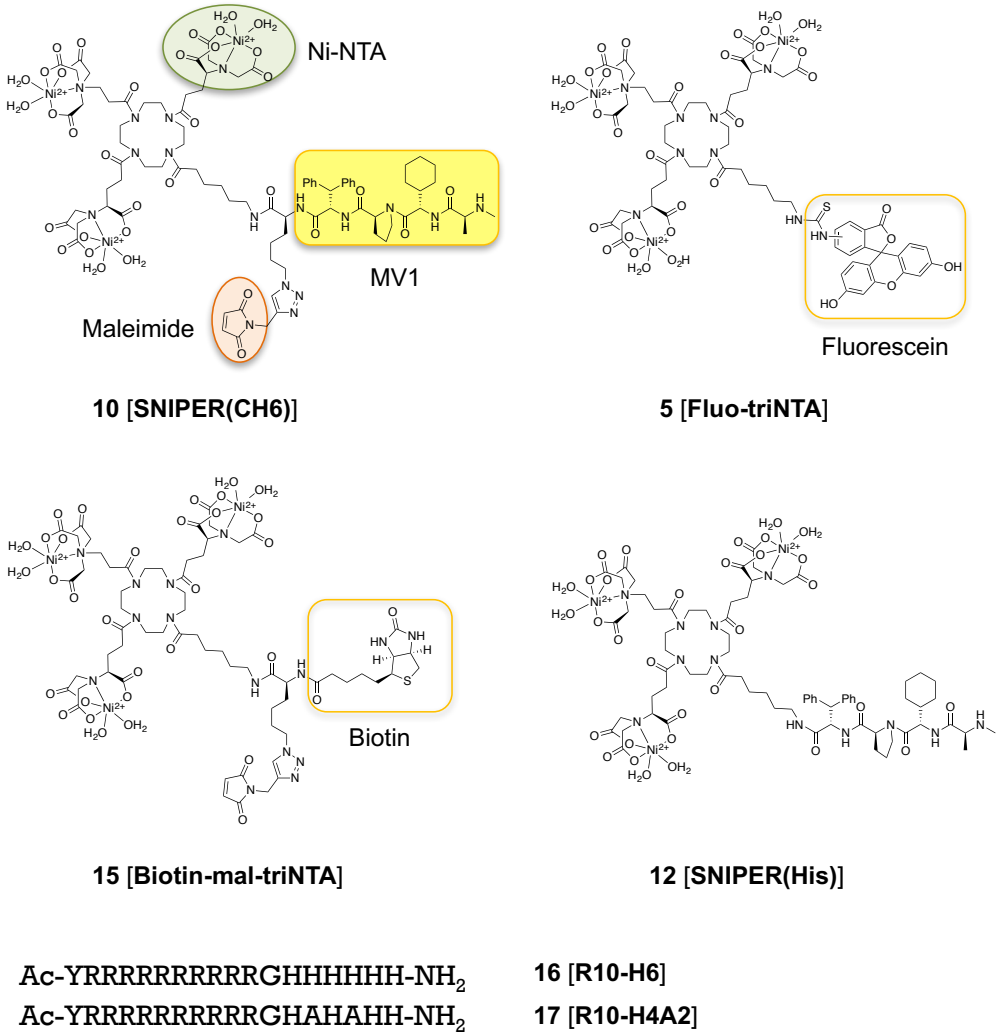


Figure 2. Delivery of **10** to ubiquitylate and degrade CH6-tagged POI. To deliver **10** into cells, we formed a **10**-carrier peptide consisting of a His-tag and a cell penetrating peptide (CPP) complex. This complex is thought to penetrate the plasma membrane efficiently. In the cytosol, **10** is released from the carrier peptide and forms another complex with CH6-tagged POI through

the covalent binding of the maleimide moiety to a Cys residue. Thereafter, POI are ubiquitylated and degraded via the UPS. See the main text for more detail.

The chemical structure of the degradation inducer and the sequence of carrier peptides developed in this study are shown in Figure 3. The degradation inducer **10** consists of three Ni-NTA moieties, which increase affinity for the His-tag;[27] a maleimide moiety, and MV1,[28] a pan-antagonist of cIAP1, cIAP2, and XIAP, as an E3 ligand. Two types of carrier peptides, **17** (**R10-H4A2**)[26] and **16** (**R10-H6**),[26] which are composed of deca-arginine (R10) and His-tags, were used. The His-tag H4A2 in the **17** carrier peptide sequence was obtained by replacing two His residues with two Ala residues. This tag was expected to exhibit reduced affinity for Ni-NTA and to facilitate dissociation of the degradation inducer within the target cells. To verify our method, we also synthesized two additional molecules in which the E3 ligand moiety was replaced with a fluorescein molecule **5** (**Fluo-triNTA**)[26] or a biotin molecule **15** (**Biotin-mal-triNTA**).[26] The molecule **12** [**SNIPER(His)**][14] without a maleimide moiety was also synthesized. The synthetic routes for these molecules are shown in the supporting information.



10 [SNIPER(CH6)] **5 [Fluo-triNTA]**

15 [Biotin-mal-triNTA] **12 [SNIPER(His)]**

Ac-YRRRRRRRRRRGHHHHHH-NH_2 **16 [R10-H6]**

Ac-YRRRRRRRRRRGHAHAHH-NH_2 **17 [R10-H4A2]**

Figure 3. Structures of the molecules used in this study.

First, we analyzed whether the carrier peptide was able to transport compound **5** into HT1080 cells. It was found that **5** was not taken into the cells in the absence of a carrier peptide **17** after a three-hour incubation because of the high polarity of the Ni-NTA moiety. On the other hand, fluorescence was observed within the cells when **5** formed a complex with **17** (Figure S1), indicating that the carrier peptide delivered **5** into the cells as expected.

Next, a biotin pull-down assay was performed using compound **15** to confirm that **15** dissociates from the carrier peptide and the maleimide moiety in **15** is able to covalently bind to the CH6-tagged POI in living cells. HT1080 cells constitutively expressing 2×(CH6)-FLAG-CRABP-II were treated with 3-μM **10**/**17** complex or 3-μM **15**/**17** complex and the cell lysate was subjected to a biotin pull-down assay with streptavidin beads. As shown in Figure 4, 2×(CH6)-FLAG-CRABP-II protein was not pulled down when the cells were treated with **10**/**17** (lane 2). On the other hand, treatment with **15**/**17** resulted in biotinylation of the 2×(CH6)-FLAG-CRABP-II protein, which was reflected in precipitation with streptavidin beads (lane 3). These observations suggested that **15** is released from the carrier peptide and forms a covalent bond with CH6-tagged CRABP-II.

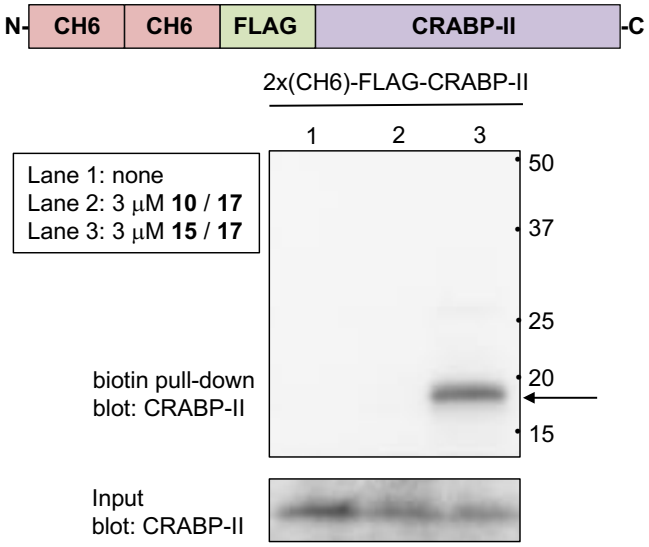


Figure 4. Compound **15** covalently attached to the 2×(CH6)-FLAG-CRABP-II protein in living cells. HT1080 cells constitutively expressing 2×(CH6)-FLAG-CRABP-II were treated with vehicle alone (lane 1), 3-μM **10**/**17** complex (lane 2), and 3-μM **15**/**17** complex (lane 3) for 2 h.

Then, the cells were harvested and lysed for a biotin pull-down assay followed by immunoblotting with anti-CRABP-II antibody. The arrow shows biotinylated 2×(CH6)-FLAG-CRABP-II protein precipitated with streptavidin agarose.

Then, we evaluated the effects of compound **10** on 2×(CH6)-FLAG-CRABP-II protein degradation in HT1080 cells (Figure 5). As shown in Figure 5a, treatment with carrier peptides alone did not affect the expression level of 2×(CH6)-FLAG-CRABP-II protein (Figure 5, lanes 4 and 5). A reduced level of the protein was observed in the cells treated with 3-μM **10**/**17** complex while the use of a combination of **10** and **16** also resulted in protein degradation (Figure 5a, lanes 2 and 3), and there were no differences in activity between **17** and **16**. However, compound **12** without the maleimide moiety did not promote the degradation of the protein, indicating that covalent binding greatly influences degradation of POI (Figure 5b, lane 2). Compound **10**-induced degradation of the 2×(CH6)-FLAG-CRABP-II protein was blocked by the addition of a proteasome inhibitor (MG132 or carfilzomib), but a lysosome inhibitor (bafilomycin A1) did not suppress the protein degradation (Figure 5b, lanes 4-9). These findings indicate that **10** induces the proteasomal degradation of the 2×(CH6)-FLAG-CRABP-II protein when delivered into cells by **16** and **17**.

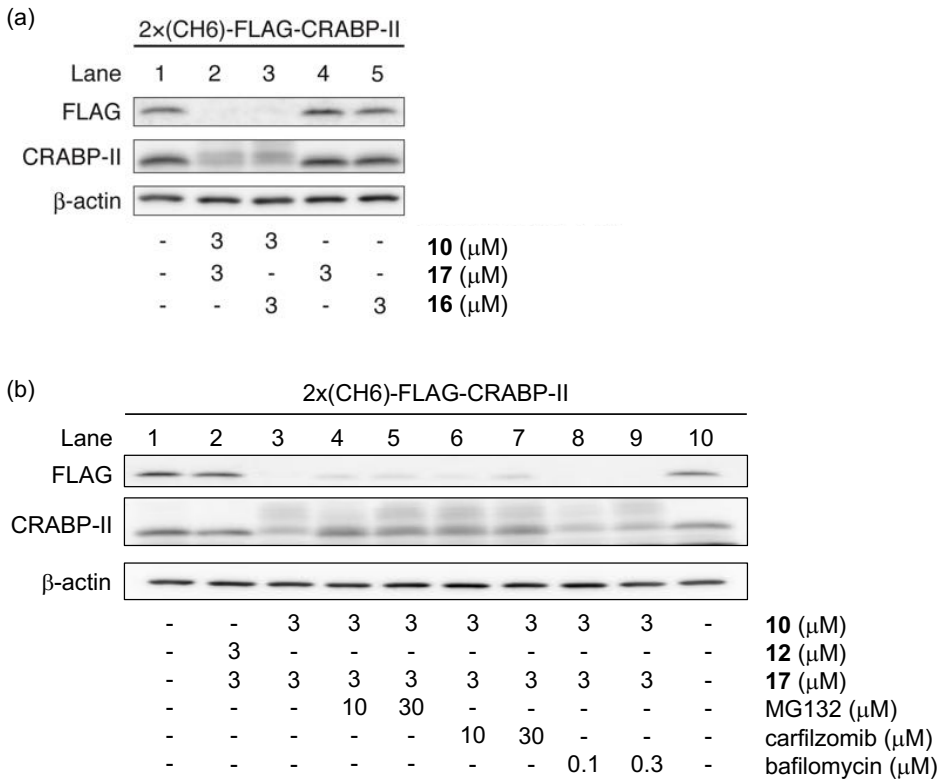


Figure 5. Degradation of 2×(CH6)-FLAG-CRABP-II by **10**. (a) HT1080 cells constitutively expressing 2×(CH6)-FLAG-CRABP-II were treated with 3-μM **10**/carrier peptide complex or carrier peptide alone for 6 h. (b) The cells were treated with 3 μM **12/17** or **10/17** complex together with the indicated doses of MG132, carfilzomib, or bafilomycin A1 for 6 h. The lysates were analyzed with the indicated antibodies.

Treatment with **10/16** complex also resulted in the degradation of the 2×(CH6)-2×FLAG-Smad2 protein (Figure 6, lanes 2 and 5). The reduction in the protein level induced by **10** was abrogated by co-treatment with MG132 (Figure 6, lanes 3, 4, 6, and 7), suggesting that the 2×(CH6)-2×FLAG-Smad2 protein is also degraded via the proteasome.

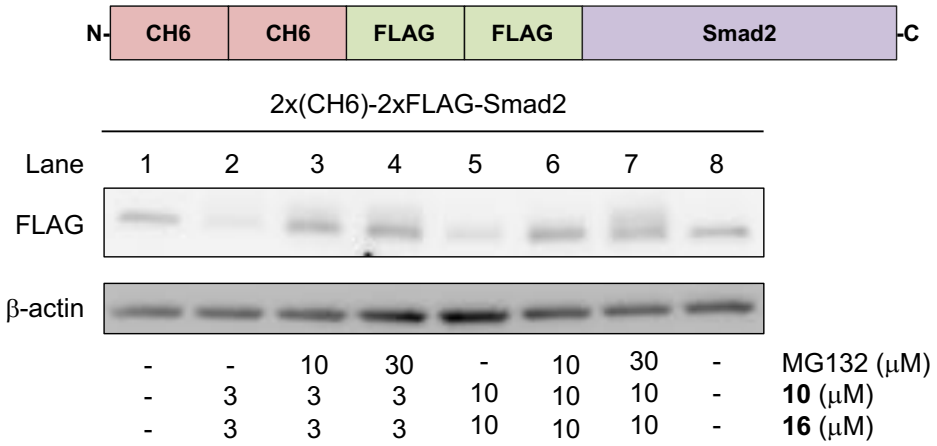


Figure 6. Degradation of the 2×(CH6)-2×FLAG-Smad2 protein by **10**/**16** complex in a proteasome activity-dependent manner. HT1080 cells constitutively expressing the 2×(CH6)-2×FLAG-Smad2 protein were treated with the indicated doses of **10**/**16** complex in the presence or absence of 10 μM or 3 μM MG132 for 6 h. The lysates were analyzed with the indicated antibodies.

Finally, to confirm the safety of **10** and the carrier peptides, we subjected these compounds to cytotoxicity tests (Figure 7). HT1080 cells were treated with each compound at concentrations of 1, 3, and 10 μM for 24 h. Compounds **10**, **17**, **16** discrete molecules, and the compound **10**/carrier peptide complexes did not exhibit marked cytotoxicity, even at a concentration of 10 μM.

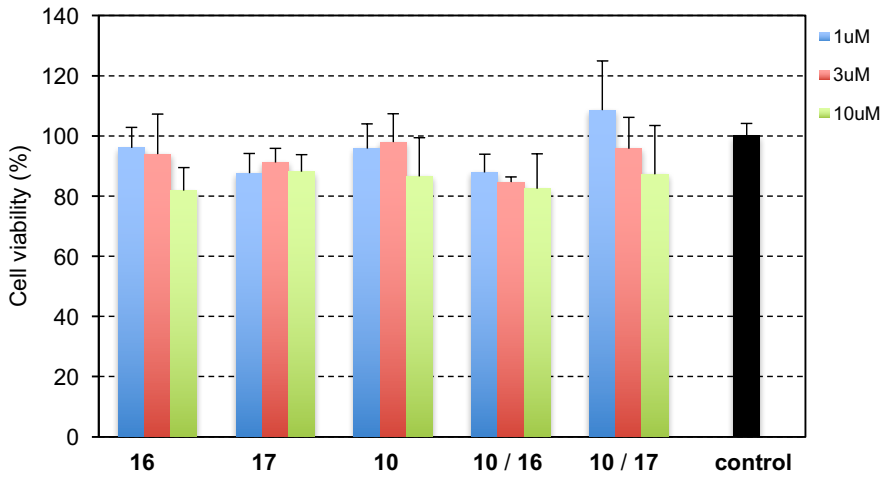


Figure 7. The compound **10**/carrier peptide complexes are not cytotoxic at the effective concentration. HT1080 cells were treated with **10**, carrier peptides, and their complexes at indicated concentrations. After 24 h, cell viability was assessed. 100% cell viability was calculated from the wells without carrier peptides or **10** (control).

Conclusions

In summary, we have successfully developed a protein-knockdown method for CH6-tagged POI using a combination of carrier peptides and **10**. The compound **10**/carrier peptide complexes were capable of inducing the degradation of intracellular CH6-tagged CRABP-II and Smad2 proteins. Our system could be an effective screening tool for finding proteins that are susceptible to degradation via the UPS, for investigating the protein function and cellular output when forced selective ubiquitylation of POI is induced, and for identifying target proteins for drug discovery.

Experimental Section

General.

Chemical reagents and solvents were purchased from *Aldrich Inc.*, *Tokyo Chemical Industry Co.,Ltd.*, *Wako Pure Chemical Industries,Ltd.*, *Kanto chemical co.,inc.*, *Watanabe Chemical Industries, LTD.* and *Quanta BioDesign, Ltd.* and used without purification. ^{1}H NMR spectra were obtained on a *Varian* Mercury (400 MHz). High-resolution mass spectra were recorded with *SHIMAZU* LCMS-IT-TOF spectrometer. The purified peptides and compounds were characterized using 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems/MDS SCIEX), and liquid chromatography-mass spectrometry-ion trap-time-of-flight (LCMS-IT-TOF) spectroscopy (Shimadzu). Analytical thin-layer chromatography was performed on *Merck* Silica Gel F_{254}. The purity of synthesized compounds was determined by analytical RP-HPLC using a Discovery® Bio Wide Pore C18 column (25 cm x 4.6 mm; solvent A:0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 1.0 mL・ml^{-1}, gradient: 10-60% gradient of solvent B over 30 min). All synthesized compounds were obtained with purity ≥95%.

Synthesis and purification of peptides.

The peptides were synthesized using solid phase methods on NovaPEG Rink amide resin following the standard Fmoc chemistry. The following describes a representative coupling and deprotection cycle at a 25 μmol scale. First, 65 mg NovaPEG Rink amide resin (loading: 0.5 mmol/g) was soaked for 1 h in CH_2Cl_2. After the resin had been washed with DMF, Fmoc-amino acid (4 Eq) and HBTU (4 Eq) dissolved in 1.0 ml NMP were added to the resin. Then, DIPEA (8 Eq) and HOBt (1.0 ml, 0.1 M solution in NMP) were added for the coupling reaction. Deprotection was carried out using 20% piperidine in NMP (2 ml). After the peptide synthesis, the resin was

suspended in cleavage cocktail (1.9 ml TFA, 50 μl water, 50 μl TIPS; final concentration: 95% TFA, 2.5% water, 2.5% TIPS) for 3 h at rt. The TFA solution was evaporated to a small volume under a stream of N_2 and dripped into cold ether to precipitate the peptides. The dried crude peptides were dissolved in 2 ml of 50% acetonitrile in water and then purified by reversed-phase HPLC using a Discovery® Bio Wide Pore C18 column (25 cm x 21.2 mm). After being purified, the peptide solutions were lyophilized. Peptide purity was deterimined by analytical HPLC using a Discovery® Bio Wide Pore C18 column (25 cm x 4.6 mm; solvent A:0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 1.0 mL・ml^{-1}, gradient: 10-60% gradient of solvent B over 30 min), and the peptides were characterized by matrix-assisted laser desorption/ionization mass spectrometry.

Reagents

DMEM, and Bafilomycin A1 were purchased from Sigma-Aldrich. Carfilzomib (LC Laboratories) and MG132 (Peptide Institute) were purchased. The peroxidase-conjugated anti-FLAG (M2) monoclonal antibody and anti-β-actin (AC-74) monoclonal antibody were from Sigma-Aldrich. The anti-CRABP-II polyclonal antibody was form BETHYL.

Cell culture condition and transfection.

HT1080 cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), and penicillin and streptomycin mixture at 37 °C in a humidified atmosphere of 5% CO_2 in air. HT1080 cells were transfected with the expression vectors for CH6-tagged POI using

Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 48 h, the cells were selected with 500 μg/ml G418 (Roche) for 2 weeks.

Formation of SNIPER-carrier peptide complex.

To load Ni^{2+} to NTA moiety of SNIPERs and its derivatives, **10**, **12**, **15**, and **5** were incubated with 3 equivalent of $NiCl_2$ in PBS (-) for 2 h. Then, the reactant was incubated with 1 equivalent of carrier peptide for 10 min before addition to the cell culture.

Fluorescence imaging.

HT1080 cells were seeded onto glass bottom dish (Greiner Bio-one) (10,000 cells/dish) and incubated overnight in 2 ml of DMEM containing 10% FBS. The medium was replaced with fresh one, and compound solution was applied to dish. After the cells had been incubated for 3 h, the medium was removed and the cells were washed with DMEM containing 10% FBS in case of washing the cells, and the intracellular distribution of the complexes was observed by Fluorescence microscope. The observations were performed using a BZ-9000 (Keyence) equipped with a 40× objective lens.

Western blotting.

Cells were treated as described in Figure legends, washed with PBS (-), lysed in SDS lysis buffer (1% SDS, 0.1M Tris-HCl and 10% Glycerol, pH 7.4) and boiled for 10 min. Protein concentrations

were determined using BCA Protein Assay Kit (Thermo Fisher Scientific) and normalized by total protein concentration in each lysate. After boiling for 5 min with Laemmli buffer, each lysate was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 5-20% gradient gel and transferred onto PVDF membrane. After blocking with TBS-T (25 mM Tris-HCl, 137 mM NaCl, 2.68 mM KCl and 0.1% Tween20, pH 7.4) containing 5% skim milk, the membrane was probed with the antibodies as described in Figure legends. The immunoblots were visualized by enhanced chemiluminescence with Clarity™ Western ECL Substrate (BIO-RAD).

Biotin pull-down assay.

HT1080 cells were transiently transfected with pcDNA3-2×(CH6)-FLAG-CRABP-II in 6-well plate. The following day, the cells were trypsinized and divided into 3 wells. 48 h after transfection, cells were treated as described in Figure legend, harvested, and lysed with SDS lysis buffer. After boiling for 10 min, the lysate was diluted 10 times with pull-down buffer (0.3 M NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% TritonX-100) and incubated with Streptavidin-Agarose (Upstate Biotech) at 4 °C for 3 h. The agarose beads were washed with pull-down buffer 4 times, eluted with 2×Laemmli buffer, and then subjected to SDS-PAGE followed by Western blot analysis.

Cytotoxicity.

HT1080 cells were seeded onto 96-well culture plate (5,000 cells/well) and incubated for 24 h in DMEM containing 10% FBS. Compound solution in fresh medium was added at each concentration (1-30 μM). After 24 h, cell viability was evaluated using cell counting kit-8

(DOJINDO) following to the manufacture's protocol. 100% cell viability was calculated from the wells without peptides **16**, **17** and **10**. The results were presented as the mean and standard error values obtained from three independent cultures.

Supporting Information

Experimental data for the ligands and peptides, and construction of expression plasmids. This material is available free of charge via the Internet at http:// pubs.acs.org.

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Author Contributions

‡These authors contributed equally.

T.H., M.N. and Y.D. designed the research and wrote the paper. K.O., T.M., T.S., and M.K. performed the experiments and analyzed results. All authors discussed the results and commented on the manuscript.

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ABBREVIATIONS USED

CRABP-II, cellular retinoic acid-binding protein II; CPP, cell penetrating peptide; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; FBS, fetal bovine serum; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; IAP, inhibitors of apoptosis proteins; MeCN, acetonitrile; Ni-NTA, nickel-nitrilotriacetic acid; NMP, *N*-methyl-2-pyrrolidone; POI, protein of interest; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; UPS, ubiquitin-proteasome system

Authors will release the atomic coordinates and experimental data upon article publication.

REFERENCES

1. Wilson, R. C.; Doudna, J. A. Molecular mechanisms of RNA interference. *Annu. Rev. Biophys.* **2013**, *42*, 217-239.

2. Hsu, P. D.; Lander, R. S.; Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **2014**, *157*, 1262-1278.

3. Guan, C.; Ye, C.; Yang, X.; Gao, J. A review of current large-scale mouse knockout efforts. *Genesis* **2010**, *48*, 73-85.

4. Kos, C. H. *Nutr. Rev.* Cre/loxP system for generating tissue-specific knockout mouse models. **2004**, *62*, 243-246.

5. Raina, K.; Lu, J.; Qian, Y.; Altieri, M.; Gordon, D.; Rossi, A. M. K.; Wang, J.; Chen, X.; Dong, H.; Siu, K.; Winkler, J. D.; Crew, A. P.; Crews, C. M.; Coleman, K. G. PROTAC-induced BET protein degradation as a therapy for castration-resistant prostate cancer. *Proc. Natl. Acad. Sci.* **2016**, *113*, 7124-7129.

6. Sakamoto, K. M.; Kim, K. B.; Verma, R.; Ransick, A.; Stein, B.; Crews, C. M.; Deshaies, R. J. Development of Protacs to target cancer-promoting proteins for ubiquitination and degradation. *Mol. Cell. Proteomics* **2003**, *2*, 1350-1358.

7. Puppala, D.; Lee, H.; Kim, K. B.; Swanson, H. I. Development of an aryl hydrocarbon receptor antagonist using the proteolysis-targeting chimeric molecules approach: a potential tool for chemoprevention. *Mol. Pharmacol.* **2008**, *73*, 1064-1071.

8. Bondeson, D. P.; Mares, A.; Smith, I. E.; Ko, E.; Campos, S.; Miah, A. H.; Mulholland, K. E.; Routly, N.; Buckley, D. L.; Gustafson, J. L.; Zinn, N.; Grandi, P.; Shimamura, S.; Bergamini, G.; Faelth-Savitski, M.; Bantscheff, M.; Cox, C.; Gordon, D. A.; Willard, R. R.; Flanagan, J. J.; Casillas, L. N.; Votta, B. J.; den Besten, W.; Famm, K.; Kruidenier, L.; Carter, P. S.; Harling, J. D.; Churcher, I.; Crews, C. M. Catalytic in vivo protein knockdown by small-molecule PROTACs . *Nat. Chem. Biol.* **2015**, *11*, 611-617.

9. Lu, J.; Qian, Y.; Altieri, M.; Dong, H.; Wang, J.; Raina, K.; Hines, J.; Winkler, J. D.; Crew, A.

P.; Coleman, K.; Crews, C. M. Hijacking the E3 ubiquitin ligase cereblon to efficiently target BRD4. *Chem. Biol.* **2015**, *22*, 755-763.

10. Winter, G. E.; Buckley, D. L.; Paulk, J.; Roberts, J. M.; Souza, A.; Dhe-Paganon, S.; Bradner, J. E. DRUG DEVELOPMENT. Phthalimide conjugation as a strategy for in vivo target protein degradation. *Science* **2015**, *348*, 1376-1381.
11. Toure, M.; Crews, C. M. Small-molecule PROTACS: new approaches to protein degradation. *Angew. Chem., Int. Ed.* **2016**, *55*, 1966-1973.
12. Zengerle, M.; Chan, K.-H.; Ciulli, A. Selective small molecule induced degradation of the BET bromodomain protein BRD4. *ACS Chem. Biol.* **2015**, *10*, 1770-1777.
13. Lai, A. C.; Toure, M.; Hellerschmied, D.; Salami, J.; Jaime-Figueroa, S.; Ko, E.; Hines, J.; Crews, C. M. Modular PROTAC design for the degradation of oncogenic BCR-ABL. *Angew. Chem. Int. Ed.* **2015**, *55*, 807-810.
14. Itoh, Y.; Ishikawa, M.; Naito, M.; Hashimoto, Y. Protein knockdown using methyl bestatin-ligand hybrid molecules: design and synthesis of inducers of ubiquitination-mediated degradation of cellular retinoic acid-binding proteins. *J. Am. Chem. Soc.* **2010**, *132*, 5820-5826.
15. Demizu, Y.; Okuhira, K.; Motoi, H.; Ohno, A.; Shoda, T.; Fukuhara, K.; Okuda, H.; Naito, M.; Kurihara, M. Design and synthesis of estrogen receptor degradation inducer based on a protein knockdown strategy. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 1793-1796.
16. Okuhira, K.; Ohoka, N.; Sai, K.; Nishimaki-Mogami, T.; Itoh, Y.; Ishikawa, M.; Hashimoto, Y.; Naito, M. Specific degradation of CRABP-II via cIAP1-mediated ubiquitylation induced by hybrid molecules that crosslink cIAP1 and the target protein. *FEBS Lett.* **2011**, *585*, 1147-1152.

17. Okuhira, K.; Demizu, Y.; Hattori, T.; Ohoka, N.; Shibata, N.; Nishimaki-Mogami, T.; Okuda, H.; Kurihara, M.; Naito, M. Development of hybrid small molecules that induce degradation of estrogen receptor-alpha and necrotic cell death in breast cancer cells. *Cancer Sci.* **2013**, *104*, 1492-1498.

18. Ohoka, N.; Nagai, K.; Hattori, T.; Okuhira, K.; Shibata, N.; Cho, N.; Naito, M. Cancer cell death induced by novel small molecules degrading the TACC3 protein via the ubiquitin-proteasome pathway. *Cell Death Dis.* **2014**, *5*, e1513.

19. Okuhira, K.; Demizu, Y.; Hattori, T.; Ohoka, N.; Shibata, N.; Kurihara, M.; Naito, M. Molecular design, synthesis, and evaluation of SNIPER(ER) that induces proteasomal degradation of ERα. *Methods Mol. Biol.* **2016**, *1366*, 549-560.

20. Ohoka, N.; Okuhira, K.; Ito, M.; Nagai, K.; Shibata, N.; Hattori, T.; Ujikawa, O.; Shimokawa, K.; Sano, O.; Koyama, R.; Fujita, H.; Teratani, M.; Matsumoto, H.; Imaeda, Y.; Nara, H.; Cho, N.; Naito, M. In vivo knockdown of pathogenic proteins via specific and nongenetic IAP-dependent protein erasers (SNIPERs). *J. Biol. Chem.* **2017**, *292*, 4556-4570.

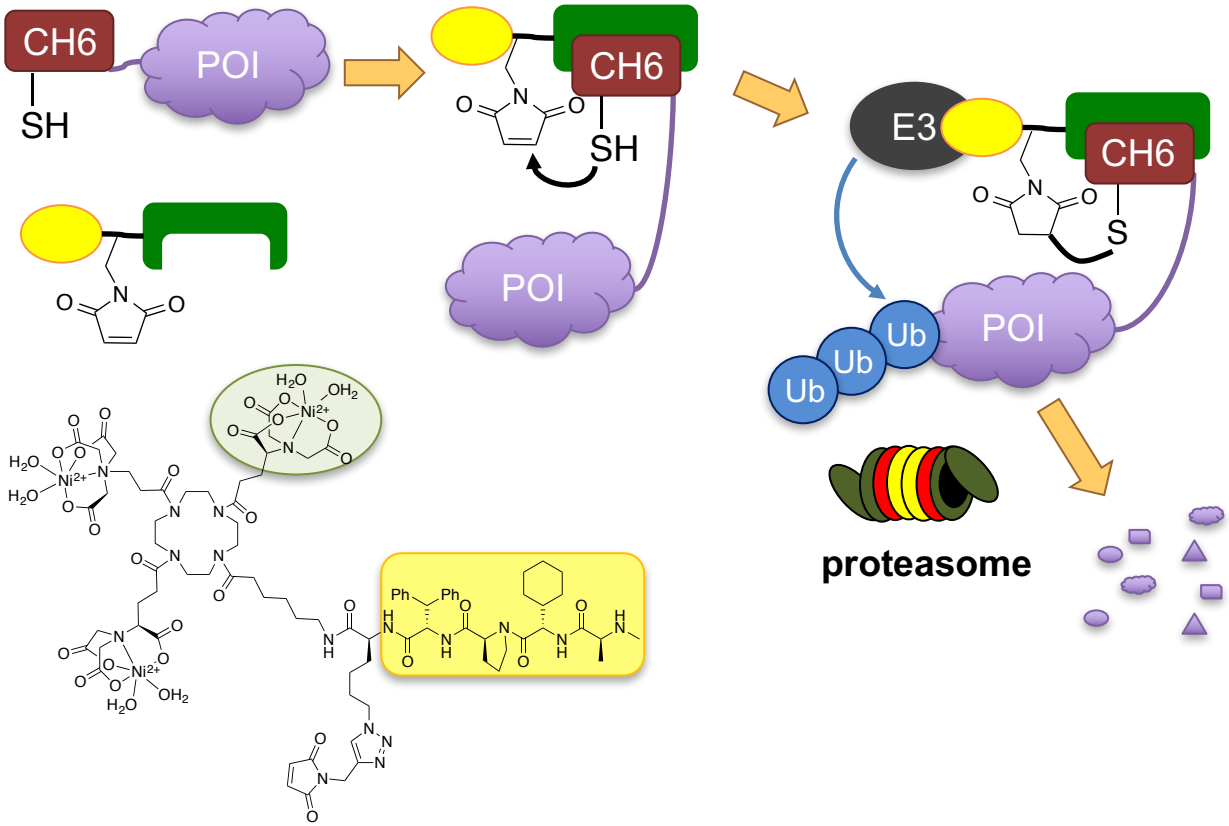
21. Okuhira, K.; Shoda, T.; Omura, R.; Ohoka, N.; Hattori, T.; Shibata, N.; Demizu, Y.; Sugihara, R.; Ichino, A.; Kawahara, H.; Itoh, Y.; Ishikawa, M.; Hashimoto, Y.; Kurihara, M.; Itoh, S.; Saito, H.; Naito, M. Targeted degradation of proteins localized in subcellular compartments by hybrid small molecules. *Mol. Pharmacol.* **2017**, *91*, 159-166.

22. Buckley, D. L.; Raina, K.; Darricarrere, N.; Hines, J. ; Gustafson, J. L. ; Smith, I. E. ; Miah, A. H. ; Harling, J. D. ; Crews, C. M. HaloPROTACS: Use of small molecule PROTACs to induce degradation of HaloTag fusion proteins. *ACS Chem. Biol*. **2015**, *10*, 1831-1837.

23. Tomoshige, S.; Naito, M.; Hashimoto, Y.; Ishikawa, M. Degradation of HaloTag-fused nuclear proteins using bestatin-HaloTag ligand hybrid molecules. *Org. Biomol. Chem.* **2015**,

13, 9746-9750.

24. Wieneke, R.; Labòria, N.; Rajan, M.; Kollmannsperger, A.; Natale, F.; Cardoso, M. C.; Tampé, R. Live-cell targeting of His-tagged proteins by multivalent N-nitrilotriacetic acid carrier complexes. *J. Am. Chem. Soc.* **2014**, *136*, 13975-13978.
25. Chen, Y.; Clouthier, C. M.; Tsao, K.; Strmiskova, M.; Lachance, H.; Keillor, J. W. Coumarin-based fluorogenic probes for no-wash protein labeling. *Angew. Chem. Int. Ed.* **2014**, *53*, 13785-13788.
26. Uchinomiya, S.; Nonaka, H.; Wakayama, S.; Ojida, A.; Hamachi, I. In-cell covalent labeling of reactive His-tag fused proteins. *Chem. Commun.* **2013**, *49*, 5022-5024.
27. Lata, S.; Reichel, A.; Brock, R.; Tampé, R.; Piehler, J. High-affinity adaptors for switchable recognition of histidine-tagged proteins. *J. Am. Chem. Soc.* **2005**, *127*, 10205-10215.
28. Itoh, Y.; Ishikawa, M.; Kitaguchi, R.; Okuhira, K.; Naito, M.; Hashimoto, Y. Double protein knockdown of cIAP1 and CRABP-II using a hybrid molecule consisting of ATRA and IAPs antagonist. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4453-4457.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