Communication

Light-induced protein degradation with photo-caged PROTACs

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Light-induced protein degradation with photo-caged PROTACs

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Supporting Information Placeholder

ABSTRACT: Induction of protein degradation is emerging as a powerful strategy to modulate protein functions and alter cellular signaling pathways. Proteolysis-targeting chimeras (PROTACs) have been used to degrade a range of diverse proteins *in vitro* and *in vivo*. Here we present a type of photo-caged PROTACs (pc-PROTACs) to induce degradation activity with light. Photo-removable blocking groups were added to a degrader of Brd4, and the resulting molecule pc-PROTAC1 showed potent degradation activity in live cells only after light irradiation. Furthermore, this molecule efficiently degraded Brd4 and induced expected phenotypic changes in zebrafish. Additionally, this approach was successfully applied to construct pc-PROTAC3 of BTK. Thus a general strategy to induce protein degradation with light was established to augment the chemists' toolbox to study disease-relevant protein targets.

Selective degradation of proteins rather than direct inhibition of protein activity emerges as a novel and powerful strategy to modulate and study the functions of proteins *in vitro*[1] and *in vivo*[2], and this approach has shown great potential to treat various diseases in patients[3]. Small molecule degraders, such as PROTACs, coined by Sakamoto, Crews and Deshaies in 2001[4], degrade proteins of interest via the ubiquitin-proteasome system. A wide range of PROTACs have been developed to degrade important proteins, such as Brd4[1,5], FKBP12[1,2b], and BTK[6]. The first oral PROTAC (ARV-110) targeting androgen receptor has recently progressed into a phase I clinical study[7].

Control of small molecule probe's activity with higher precision has been the Holy Grail of chemical biology and drug discovery. Light with high spatiotemporal resolution has been widely used in neurobiology, chemical biology[8] and disease treatment[9] through the light-controlled release of active small molecule modulators. We envisioned to incorporate photo-control groups into PROTACs and developed a general strategy to switch on the degradation activity of PROTACs by light (Figure 1a). Herein, we report the discovery of photo-caged PROTACs (pc-PROTACs), which are inactive without irradiation with light, and upon irradiation, can quickly release active PROTACs to efficiently degrade the target proteins and to induce desired phenotypes in both live cells and zebrafish. Around the submission of our manuscript , three preprints of photoactivatable PROTACs were reported online, utilizing photoswitchable diazo compounds[10] or a photocleavable coumarin group[11].

A PROTAC molecule is composed of three basic components: (1) a ligand to recruit E3 ubiquitin ligase, (2) a ligand to bind a target protein, and (3) a linker to connect the two recruiting ligands. We selected bromodomain and extraterminal (BET) protein bromodomain-containing protein 4 (Brd4) as the first target protein due to its importance as a potential therapeutic target and an elegant previous work in the literature[1,12]. The template of pc-PROTACs is based on the Bradner group's dBET1[1], where thalidomide, a ligand of E3 ligase cereblon (CRBN)[13], is linked to JQ1, a ligand of Brd4[14] (Figure 1b). dBET1 selectively and highly efficiently degrades BET proteins. The photo-control group has to serve two purposes: one is to block the formation of binding complexes between pc-PROTACs and its target protein or E3 ligase, and the other is to be cleaved upon light irradiation. 4,5-Dimethoxy-2-nitrobenzyl (DMNB) group can be efficiently cleaved upon irradiation at 365 nm and has been used in numerous cellular studies[8]. Based on the CRBN-thalidomide (PDB ID: 4CI1)[13] and Brd4-JQ1 (PDB ID: 3MXF)[14] crystal structures (Figure S1), we hypothesized that incorporation of the bulky DMNB group through the amide nitrogen of the JQ1 moiety (pc-PROTAC1) or the imide nitrogen of the thalidomide moiety (pc-PROTAC2) (Figure 1c) may hamper the binding and degradation capability of pc-PROTACs.

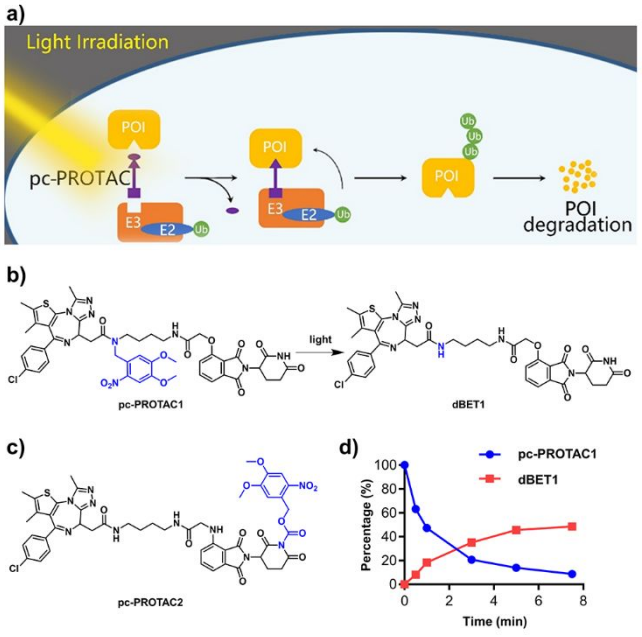

Figure 1. Release of an active PROTAC from pc-PROTAC upon

light irradiation. (a) Schematic representation of the pc-PROTAC strategy. Under light irradiation, a photo-removable group (purple circle) is cleaved from pc-PROTAC and an active PROTAC is released to degrade the protein of interest (POI). (b) Uncaging reaction of pc-PROTAC1. (c) Chemical structure of pc-PROTAC2. (d) Photo-induced release efficiency of pc-PROTAC1 to generate dBET1.

These two pc-PROTACs were synthesized through modified literature routes[1] (Schemes S1, S2). Ultraviolet (UV) spectra of both compounds showed significantly enhanced absorption in the region around 365 nm (Figure S2a). The photolysis of pc-PROTACs and generation of dBET1 upon irradiation (365 nm, 3 mW/cm^2) were monitored by high-performance liquid chromatography. Both pc-PROTAC1 and pc-PROTAC2 disappeared quickly with half-times ($t_{1/2}$) of 60 s and 105 s, respectively (Figures 1d, S3b). However, pc-PROTAC1 generated about 50% of the desired product dBET1 (Figures 1d, S2b), but pc-PROTAC2 failed to produce appreciable amount of any major product (Figures S3c). The stability of pc-PROTAC1 in the dark was also examined in the PBS buffer solution for 24 h. Approximately 88% of pc-PROTAC1 remained intact and no dBET1 was released (Figure S4). Therefore, pc-PROTAC1 was selected for subsequent studies.

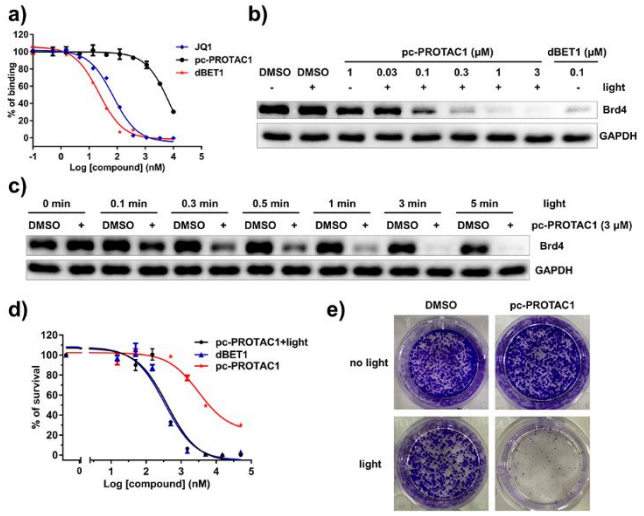

Figure 2. *In vitro* experiments of pc-PROTAC1 with or without irradiation. (a) Binding affinity of compounds to Brd4 bromodomain 1. (b) Brd4 levels were reduced in a dose-dependent manner upon irradiation. (c) Results of western blot assay of Brd4 levels in Ramos cells at various irradiation times. (d) Results of cell viability assay in Namalwa cells. (e) Results of colony-forming assay in HUH7 Cells with pc-PROTAC1 (5 μM).

As expected, the binding affinity of pc-PROTAC1 to Brd4 was greatly reduced with an IC_{50} value of 7.6 μM, while the IC_{50} values of JQ1 and dBET1 were 71 nM and 22 nM, respectively (Figure 2a), demonstrating that introduction of the bulky DMNB group at the JQ1 moiety efficiently reduced the binding ability of pc-PROTAC1 to Brd4 by more than 100-fold. To determine whether pc-PROTAC1 can degrade Brd4 under various conditions, Ramos cells were incubated with various concentrations of the compound for 1 h and the quantity of Brd4 was measured 12 h later. Consistent with a substantially weaker binding affinity, 3 μM pc-PROTAC1 did not show much appreciable degradation of Brd4 in the dark (Figure S5a). However, upon irradiation with UV light at 365 nm for 3 min, Brd4 was dose dependently degraded. 0.3 μM pc-PROTAC1 significantly reduced the Brd4 levels similar to the effects of 0.1 μM dBET1, and 1 μM pc-PROTAC1 almost completely degraded Brd4 with maximum degradation efficacy (D_{max}) of 93% (Figures S5b, 2b). The same degradation experiment was conducted at various irradiation times (Figure 2c). Reduction in the Brd4 protein levels was clearly observed after a brief exposure to the light at 365 nm for only 0.3 min, while longer exposure time for 3 min led to complete degradation of Brd4. The kinetics of Brd4 degradation by pc-PROTAC1 upon irradiation for 3 min was examined (Figure S6), and the data showed that Brd4 was almost completely degraded after 4 h, similar to the effect of dBET1. When cells were incubated with pc-PROTAC1 in the dark for 2 h and then briefly washed with fresh medium to remove free pc-PROTAC1, the Brd4 protein levels were still significantly degraded after the cells were irradiated (Figures S7a, S7b).

Burkitt's lymphoma cells are sensitive to Brd4 degradation[5a], hence we treated Namalwa cells with a range of concentrations of dBET1 and pc-PROTAC1 with or without irradiation to characterize the anti-proliferative activity of these compounds. Without irradiation, the anti-proliferative activity of pc-PROTAC1 (GI_{50} = 3.1 μM) was substantially weaker than that of dBET1 (GI_{50} = 0.34 μM), and at the highest concentration of 50 μM, pc-PROTAC1 was unable to completely inhibit cell proliferation (Figures 2d). However, upon irradiation, pc-PROTAC1 had a significant inhibitory effect on cell growth (GI_{50} = 0.4 μM) similar to that of dBET1. Next the 10-day colony-forming assay was performed in Brd4-sensitive hepatocellular carcinoma HUH7 cells[15] to evaluate the long-term anti-proliferative effects of pc-PROTAC1. With irradiation pc-PROTAC1 (5 μM) showed almost complete inhibition while pc-PROTAC1 without irradiation showed no effect to reduce the colony density (Figures 2e). Thus, these results demonstrated that UV light can induce pc-PROTAC1 to generate significant degradation of target protein Brd4 in the live cells and inhibit the proliferation of the tumor cells.

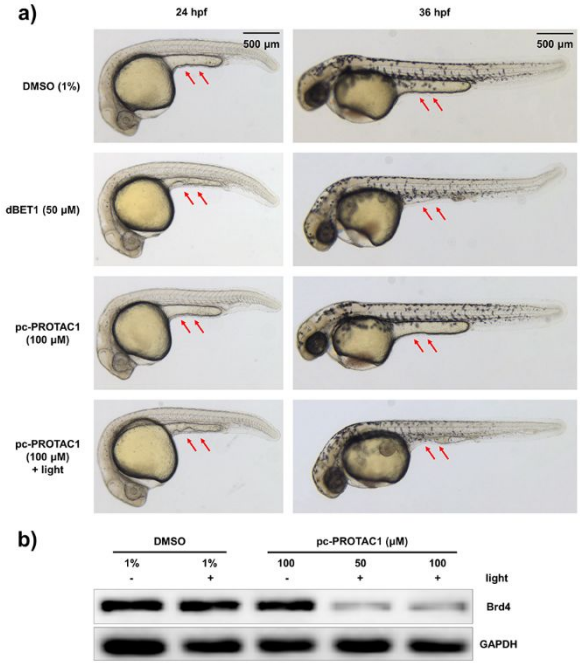

Figure 3. Degradation of endogenous Brd4 protein in zebrafish embryos. (a) Phenotype of zebrafish embryos treated by dBET1 and pc-PROTAC1 at 24 hpf and 36 hpf. (b) Expression of Brd4 protein in treated embryos examined by western blot.

Then we used zebrafish as a model organism to evaluate the *in vivo* activity of pc-PROTAC1. The biological advantages of the

zebrafish embryo model, such as *ex vivo* development and optical transparency, make it an ideal system for developmental biology studies[16] and drug discovery[17]. Brd4 protein contains three domains, BD1, BD2 and ET (Figure S8a), which are conserved among various species[18]. Specifically, amino acid sequences of the BD1 and BD2 domains, which contain binding sites of JQ1[14], are highly conserved between zebrafish and human (Figure S8b). Moreover, zebrafish CRBN also showed high sequence similarity with the human orthologue (Figure S9). In zebrafish, *brd4* is widely expressed at relatively high levels during early embryogenesis[18]. Thus, embryos at 12 hours postfertilization (hpf) were treated with pc-PROTAC1 (100 μM in embryo medium). After exposure to light at 365 nm for 10 min, embryos were incubated at 28.5 °C. Embryos treated with DMSO (1% in embryo medium) served as a blank control, embryos treated with dBET1 (50 μM in embryo medium) were used as a positive control, and embryos treated with pc-PROTAC1 (100 μM in embryo medium, but without irradiation) were used as a negative control. Embryos treated with pc-PROTAC1 with irradiation or dBET1 showed thinner yolk extension compared to that in the controls (DMSO and pc-PROTAC1 without irradiation) at 24 hpf (Figure 3a). The yolk extension of the embryos treated with pc-PROTAC1 with irradiation or dBET1 became barely observable at 36 hpf (Figure 3a), suggesting that treatment with dBET1 and uncaging of pc-PROTAC1 by light disturbed early development of the zebrafish embryos. To detect the degradation activity of pc-PROTAC1, western blot was used to assay Brd4 protein levels in the whole cell extracts of the treated embryos at 36 hpf. The results showed that Brd4 protein was significantly degraded in the embryos treated with dBET1 (Figure S10) and pc-PROTAC1 (50 or 100 μM) with irradiation (Figure 3b). These results confirmed the light induced degradation activity of pc-PROTAC1 in zebrafish.

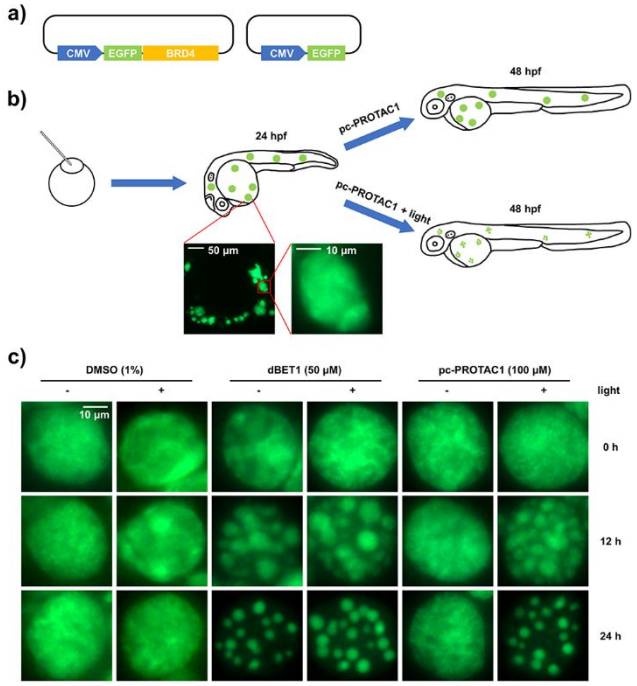

Figure 4. Degradation of EGFP-tagged Brd4 protein in zebrafish embryos. (a) Expression plasmid encoding EGFP-tagged Brd4 or EGFP only. (b) Scheme of visualizing Brd4 protein degradation in living embryos. (c) Degradation of EGFP-tagged Brd4 protein in treated embryos at 0, 12 and 24 h after treatment.

Green fluorescent protein provides a powerful means to directly visualize proteins in the biological systems, and has been utilized to detect Brd4 degradation *in vitro*[5b]. Here, an expression plasmid with a cytomegalovirus (CMV) promoter encoding an enhanced green fluorescent protein (EGFP) tagged Brd4 protein was injected into the zebrafish embryos at the single cell stage (Figures 4a, 4b), and the injected embryos were incubated at 28.5 °C until 24 hpf. The cells with the EGFP signals were distributed randomly throughout the embryos. Then, the embryos were treated with DMSO, dBET1 and pc-PROTAC1 with or without irradiation. The degradation of EGFP-Brd4 in the embryos was initially observed as early as 8-10 h after treatment with pc-PROTAC1 with irradiation or dBET1. After 12 to 24 h, most of the EGFP-Brd4 expressing cells showed substantial degradation of fluorescent protein (Figure 4c). In contrast, EGFP in embryos treated by DMSO or pc-PROTAC1 without irradiation were remained (Figure 4c). As a control, embryos injected with a plasmid encoding only EGFP were also treated as described above, and no degradation of EGFP was observed (Figure S11). Collectively, these results demonstrate that UV light can induce pc-PROTAC1 to degrade Brd4 in the zebrafish embryos.

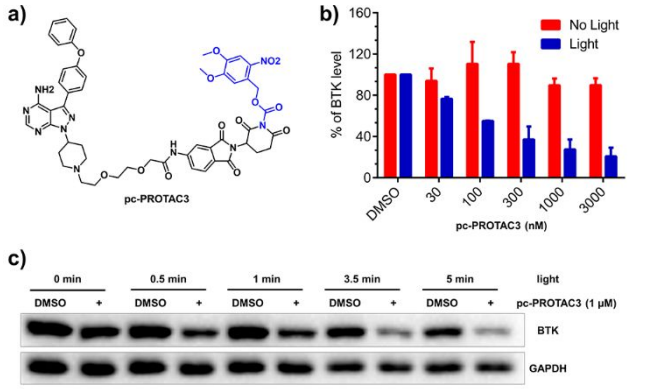

Figure 5. BTK degradation by pc-PROTAC3. (a) The chemical structure of pc-PROTAC3. (b) Upon irradiation, BTK levels were reduced by pc-PROTAC3 in a dose-dependent manner (mean ± SD). (c) Results of western blot assay of the BTK levels in Ramos cells at various irradiation times.

This light-induced degradation strategy is not restricted to Brd4 protein. pc-PROTAC3 (Figures 5a, S12a) was designed and synthesized (Scheme S3) with the bulky DMNB group connected to the imide nitrogen of MT-802 developed by the Crews' group to degrade BTK protein[6a], an important drug target[19]. pc-PROTAC3 had absorption spectrum, stability and photo-induced release efficiency similar to those of pc-PROTAC1 (Figures S12b, S12c, S12d, S12e). Inhibition potency of pc-PROTAC3 against BTK is close to that of MT-802 since both compounds share the same binding region to BTK (Figure S12f). Ramos cells were treated with various concentrations of pc-PROTAC3 and only upon light irradiation at 365 nm for 3.5 min, BTK levels were significantly reduced in a dose-dependent manner after 18h (Figures 5b, S13). Similar to pc-PROTAC1 in degradation of Brd4, the light-induced degradation of BTK by pc-PROTAC3 was influenced by irradiation time (Figure 5c).

In conclusion, we have presented a general strategy to develop photo-caged PROTACs inducible by light to degrade proteins of interest. The degradation activity of pc-PROTACs upon irradiation is comparable to that of the corresponding PROTACs in the live cells. Furthermore, light-induced protein degradation mediated by pc-PROTACs is effective in zebrafish, a simple yet powerful animal model for developmental biology and early drug screen studies. Because light can function at millisecond and submicron resolutions, we envision that this strategy will show great promise

in the studies of protein functions and may be even incorporated to treat localized diseases.

ASSOCIATED CONTENT

Supporting Information

Compound synthesis, experimental details and supporting figures are presented in the Supporting Information. The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interests.

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