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Degradation of target protein in living cells by small-molecule proteolysis inducer

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Abstract—Ubiquitin-dependent proteolysis of cellular proteins is one of the major pathways to regulate protein function post-translationally. Here we demonstrate a potentially general method of degrading any targeted proteins by the ubiquitin-dependent proteolysis in living cells, using small-molecule proteolysis inducer (SMPI).

While the use of small molecules as therapeutic agents or lead compounds in drug development process has been a core of the modern pharmaceutical industry, these cell-permeable compounds have been increasingly used as molecular probes to explore intracellular processes.[1–3] Consequently, development of small-molecule modulators of proteins has become a major task to explore protein function, particularly, in the postgenomic era. Currently, most efforts in identifying small-molecule modulators of proteins have been pharmacologically driven. Typically, these modulators control signaling events by directly binding to intracellular targets, either repressing or stimulating biological processes. Meanwhile, in living cells many important biological processes are regulated by the ubiquitin-proteasome pathway,[4–6] which is one of the major pathways to regulate protein function post-translationally.

The ubiquitin-proteasome pathway is the principle conduit for protein turnover in all eukaryotic cells. Ubiquitin-dependent proteolysis involves the assembly of a ubiquitin chain on a substrate, which targets the attached protein for degradation by the 26S proteasome.[7–9] Ubiquitin is first activated at its C-terminus by adenylation and formation of a thioester bond with the ubiquitin-activating enzyme, E1. Activated ubiquitin is subsequently transferred from E1 to a cysteine residue of a member of the family of ubiquitin-conjugating (E2) enzymes. Finally, ubiquitin is transferred from E2 enzyme to a lysine residue of a target protein, either directly or with the assistance of a ubiquitin ligase (E3). Although E2s can directly transfer ubiquitin to model substrates in vitro, most physiological ubiquitination reactions are thought to require the participation of an E3. E3s appear to be the primary source of substrate specificity in the ubiquitination cascade, as they have been shown to bind directly and specifically to substrates.[7–9] Targeting a substrate by an E3 appears to be precisely controlled by posttranslational modifications such as phosphorylation or hydroxylation, since the ubiquitin-proteasome pathway seems to be constitutively active regardless of the status of signaling cascades (i.e., on or off). Consecutive cycles of ubiquitin transfer by E2 to substrate result in the assembly of a multiubiquitin chain on the substrate, which targets it for destruction by the 26S proteasome. Previously, it has been shown that SCF (Skp1-Cullin-F-Box) complex, one of the most-studied E3 ubiquitin ligase complexes,[7–9] can be used to target a protein for ubiquitination and degradation in vitro,[10,11] using a chimeric molecule that recruits a target protein to the SCF complex. However, the approach has not been accomplished in vivo (or in living cells) due in large part to the poor membrane permeability and bioavailability of the chimerae. In addition, when the SCF recognition motif is inserted into a cellular protein, it is shown to direct the degradation of otherwise stable cellular protein both in yeast and in mammalian cells.[12]

Herein, we report for the first time the development of a cell-permeable small-molecule proteolysis inducer

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(SMPI) that exploits the E3 ubiquitin ligase pVHL (von Hippel-Lindau tumor suppressor): hypoxia-inducible factor-1α (HIF-1α) interaction,[13–16] thereby inducing ubiquitination and degradation of a target protein in living cells (Fig. 1). One domain of the SMPI contains the HIF-1α protein-derived octapeptide motif that is recognized by the pVHL E3 ubiquitin ligase complex, whereas the other domain is composed of a protein ligand. In addition, we report for the first time herein that a reversible ligand that non-covalently binds to a target protein can be used for the SMPI approach.

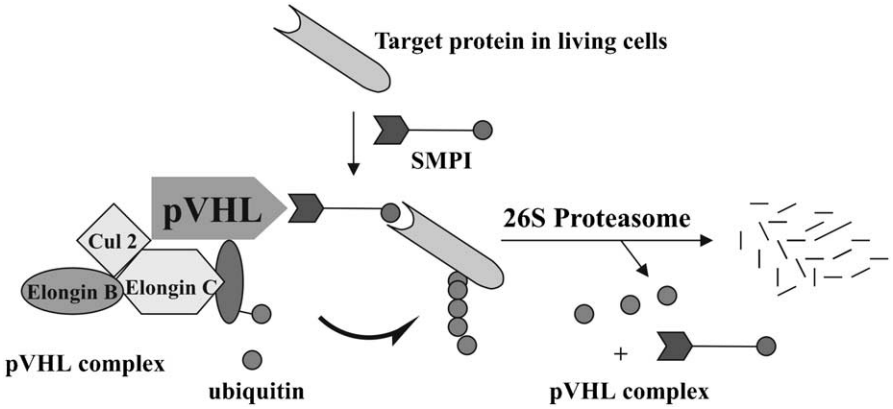

Figure 1. A cell-permeable small-molecule proteolysis inducer (SMPI) recruits target protein to the pVHL complex for ubiquitination and degradation in living cells.

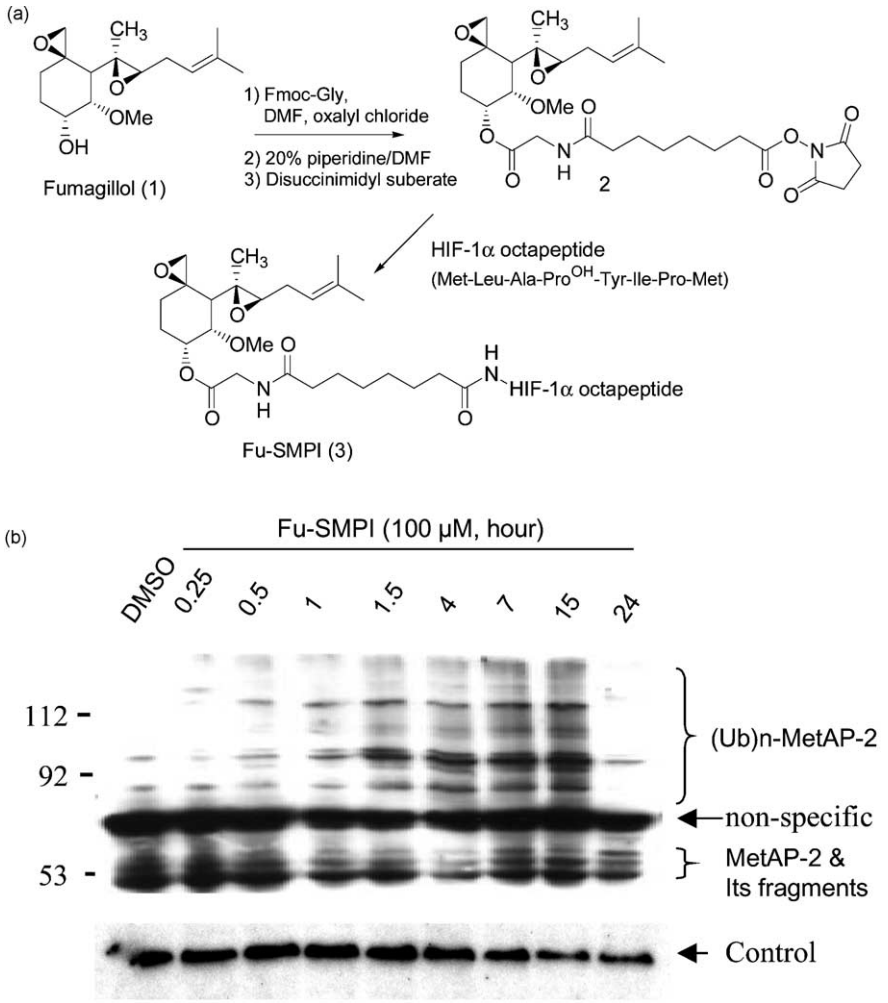

Figure 2. (a) Synthesis of fumagillol-coupled small-molecule proteolysis inducer (Fu-SMPI); (b) Western blots of lysates from Fu-SMPI-treated cells (A549) probed with anti-MetAP-2 antibody: a time-dependent accumulation of ubiquitinated MetAP-2 in lung cancer cells (A549). It should be noted that MetAP-2 is continuously synthesized in living cells throughout the incubation period, whereas all of Fu-SMPI were consumed or decomposed within 24 h, thereby observing no ubiquitinated MetAP-2 after 24 h.

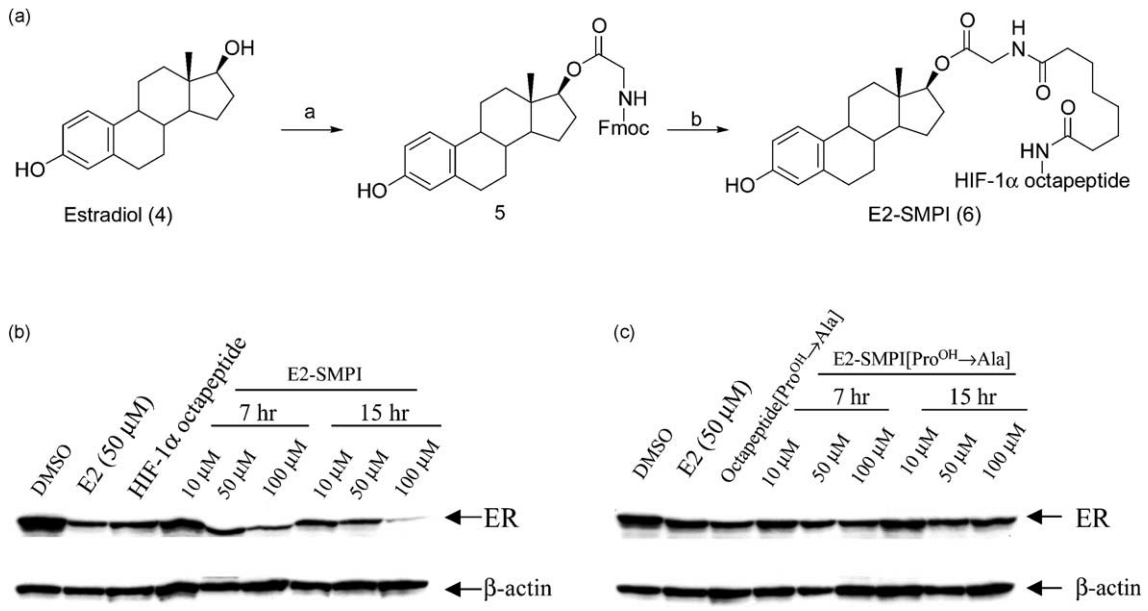

Figure 3. (a) Synthesis scheme of E2-SMPI: (a) (i) Fmoc-Gly-OH, oxalyl chloride/DMF; (ii) E2/DMAP; (b) (i) 20% Piperidine/DMF; (ii) Disuccinimidyl suberate (DSS); (iii) HIF-1α octapeptide or HIF-1α octapeptide[Pro^{OH}→Ala]. Western blots of lysates from E2-SMPI-treated MCF-7 cells probed with anti-ER antibody; (b) E2-SMPI selectively induced ER degradation, where MCF-7 breast cancer cells were treated with 10–100 μM of E2-SMPI, and incubated for 7 h or 15 h before cell lysis and immunoblotting; (c) MCF-7 cells were treated with 10–100 μM of E2-SMPI [Pro^{OH}→Ala], and incubated for 7 h or 15 h. Cells then were lysed, and immunoblotted with anti-ER antibody. All controls were incubated for 15 h.

The HIF-1α plays a key role in the adaptation of mammalian cells during low-oxygen stress,[17] and is rapidly degraded under normal oxygen concentration (or normoxic) conditions by the ubiquitin-proteasome pathway. Degradation of HIF-1α under normoxic conditions is triggered by the hydroxylation of a conserved proline residue (Pro^{564}) that is subsequently recognized by the pVHL E3 ligase, a component of ubiquitin ligase complex that mediates ubiquitination and degradation of HIF-1α.[13,14] Interestingly, it has been shown that a synthetic octapeptide derived from HIF-1α residues 561 to 568, which contains a hydroxyproline at position 564, is sufficient to interact with the pVHL.[13] Taken together, we envision that a chimeric molecule, which is composed of the synthetic HIF-1α octapeptide and a ligand of a target protein, will recruit the target protein to the E3 ligase pVHL for ubiquitination and subsequent degradation by the 26S proteasome (Fig. 1). To this end, we prepared a small-molecule proteolysis inducer, which is designed to target methionine aminopeptidase-2 (MetAP-2), by coupling fumagillol to the HIF-1α octapeptide to yield fumagillol-coupled HIF-1α octapeptide (Fu-SMPI) (Fig. 2a). Fumagillol is an active derivative of the angiogenesis inhibitor fumagillin, which selectively binds to methionine aminopeptidase-2 (MetAP-2)[18,19] that is not known to be ubiquitinated.

When lung cancer cells (A549) were treated with Fu-SMPI, ubiquitinated MetAP-2s were time-dependently accumulated, and eventually degraded (Fig. 2b).[20] Moreover, these high molecular species (multi-ubiquitinated MetAP-2s) were consistently competed away by excess fumagillol (data not shown), confirming that MetAP-2 was selectively ubiquitinated by Fu-SMPI. Given that the covalent nature of fumagillol–MetAP-2 interaction,[10] we next asked whether a reversible ligand-based SMPI induces ubiquitination and degradation of a target protein. To test this, we chose the estrogen receptor (ER) ligand estradiol (E2) and prepared E2-based ER-targeting SMPI in which a HIF-1α octapeptide was linked to estradiol using a synthetic approach similar to that of Fu-SMPI (Fig. 3a). When MCF-7 breast cancer cells were treated with E2-SMPI for 15 h, remarkably, the ER protein was completely disappeared (Fig. 3b). That degradation of the ER was mediated by the pVHL–E2-SMPI interaction was further confirmed by the treatment of E2-SMPI [Pro^{OH}→Ala], which contains a mutant HIF-1α octapeptide and therefore does not interact with the pVHL. In deed, E2-SMPI[Pro^{OH}→Ala] did not induce degradation of the ER as shown in Fig. 3c.

Taken together, these results indicate that the pVHL-mediated SMPI approach can be applied to target proteins that promote diseases. In this study, we developed a cell-permeable small-molecule proteolysis inducer that recruits a target protein for ubiquitination and degradation. Unlike conventional small-molecule ligands that must inhibit target proteins to be useful as therapeutic agents, the advantage of SMPI approach is that any protein ligands including non-functional ligands will be useful in the design of SMPI. In other words, as long as a protein ligand has the ability to interact with a target protein (i.e., either inhibitory, non-functional, or stimulatory interactions), the ligand-based SMPI is expected to ubiquitinate and degrade the target protein, given that SMPI approach is driven solely by the protein-binding ability of ligands but not by the pharmacological activity.

In summary, we report here the development of cell-permeable small-molecule proteolysis inducers (SMPIs)

that exploit the unique characteristics of the E3 ubiquitin ligase (pVHL)-mediated protein degradation pathways. The cell-permeable SMPIs selectively induced ubiquitination and degradation of target proteins in living cells. These results provide the generality of the SMPI approach to target other proteins for the ubiquitin-proteasome pathway, providing the general strategy to degrade proteins that play an important role in disease development and progression and are not considered to be readily 'drugable target'. The potential application of SMPI approach includes development of a new class of therapeutic agents with which disease-causing proteins can be destroyed through the SMPI-induced ubiquitination. Current efforts are focused on the development of HIF-1α-octapeptide-replacing non-peptide small molecules.

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20. The incubated cells with SMPI at 37 °C were lysed by lysis buffer (20 mM Tris–HCl pH 7.4, 1% Triton X-100, 5 mM EDTA, 10 μL of Protease inhibitor and 860 μL of distilled water). The protein concentrations were measured using Bio-Rad protein assay reagent. SDS-PAGE was carried out with a 8% SDS separating gel. The separating gel (2.7 mL of 30% Acrylamide/Bis-, 2.5 mL of 1.5 M Tris pH 8.8, 4.6 mL of D.D.W., 50 μL of 20% SDS, 100 μL of 10% APS and 6 μL of TEMED) and stacking gel (1.7 mL of 30% Acrylamide/Bis-, 2.5 mL of 0.5 M Tris pH 6.8, 5.6 mL of distilled water, 50 μL of 20% SDS, 100 μL of 10% APS and 10 μL of TEMED) were prepared just before running. 20 mg of protein samples were loaded from each time points and control. The electrophoresis was performed at 120 V for 2 h using a running buffer system (3.03 g of Tris–Cl, 14.41 g of Glycin, 5 mL of 20% SDS, and distilled water up to 1 L). The proteins were then transferred to PVDF membrane (Amersham) at 250 mA electric current for 2 h using a transfer buffer system (3.78 g of Tris–Cl, 18 g of Clycine, 1.85 mL 20% SDS, and distilled water up to 1 L). The membranes were washed three times with PBST buffer, were then treated with 5% skim milk for 3 h at room temperature (or overnight at 4 °C). After 2 h-incubation with the primary MetAP-2 antibody (1:250 in BSA, Zymed) at room temperature (or overnight at 4°C), the membrane was incubated with the secondary antibody (1:10,000 in 3% skim milk, Amersham) at rt for 2 h. The membranes were then washed with PBST three times. Finally, the film was developed by Western blotting detection reagents (Amersham) using a Kodak film (Kodak X-OMAT AR).