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Discovery of Small Molecules that Induce Degradation of Huntingtin

Shusuke Tomoshige,[a][b] Sayaka Nomura,[a][b] Kenji Ohgane,[a] Yuichi Hashimoto,[a] and Minoru Ishikawa[a]*

Abstract: Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by aggregation of mutant huntingtin (mHtt), and removal of toxic mHtt is expected to be an effective therapeutic approach. We designed two small hybrid molecules **1** and **2**, by linking a ligand for ubiquitin ligase (cellular inhibitor of apoptosis protein 1; cIAP1) with probes for mHtt aggregates, anticipating that these compounds would recruit cIAP1 to mHtt and induce selective degradation via the ubiquitin-proteasome system. The synthesized compounds reduced mHtt levels in HD patients' fibroblasts, and appear to be promising candidates for treatment of HD.

Neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD) and polyglutamine (polyQ) diseases, are caused by aggregates of misfolded proteins, which can be stained by fluorescent small molecules such as thioflavin T (ThT) that specifically bind to the aggregates. Among the nine polyQ diseases, Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by expansion of the CAG repeat sequence in *HTT* exon 1 gene to >35 repeats; the resulting gene encodes aggregation-prone mutant huntingtin (mHtt) with an extended N-terminal polyQ tract.[1] Due to its beta-sheet-rich structure, mHtt accumulates in neuronal cells as intracellular aggregates. These aggregates, including soluble oligomer intermediates, cause neuronal cell death, resulting in various symptoms such as motor dysfunction, that progress and lead to death within 15-20 years.[2] Several groups have reported various approaches to target the toxic mHtt aggregates, including small molecules that inhibit or accelerate aggregation of mHtt[3] or induce a molecular chaperone that suppresses the aggregation,[4] expression of 46-mer peptides that induce autophagy in order to degrade mHtt,[5] and gene silencing of mHtt.[6,7] Despite these efforts, no effective clinical treatment is yet available, because the modes of action of the chemical aggregation modulators remain unclear[8] and biological approaches have several limitations, including poor metabolic stability, limited selectivity and low permeability of reagents.[9] We believe, therefore, that novel approaches employing small molecules are required for effective treatment of HD.

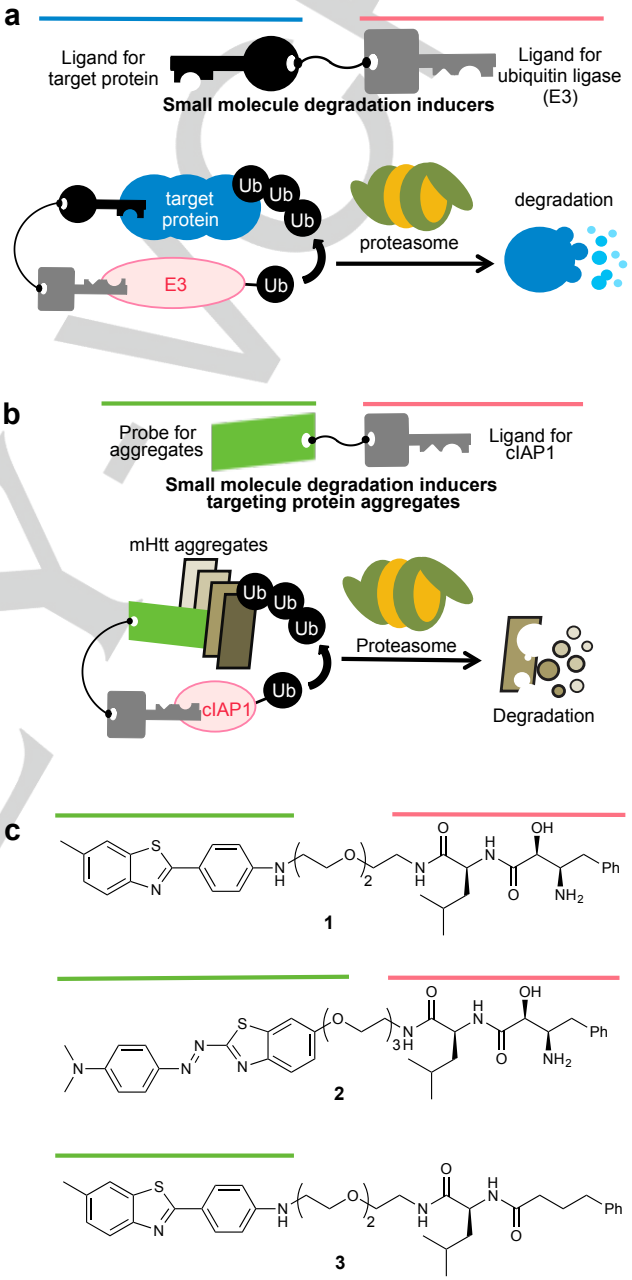

Figure 1. Small molecule-mediated protein-degradation technology and the newly designed small hybrid molecules. (**a**) Proposed mechanisms. Degradation inducers induce complex formation between target protein and ubiquitin ligase (E3), followed by ubiquitination (Ub) of target protein, and proteasomal degradation. (**b**) The concept of this study. Small hybrid molecules consisting of a probe for aggregates linked to a ligand of cIAP1 are expected to induce degradation of mHtt aggregates. (**c**) Structures of **1**-**3**.

The concept of hybrid molecules with a dual mode of action is the focus of the attention in small molecule drug discovery

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programs.[10] In recent years, a strategy has been developed for degradation of target proteins based on the use of small hybrid molecules composed of ligand for the target protein and a ligand for ubiquitin ligase (E3); these hybrid molecules recruit E3 to the target protein, leading to selective degradation *via* the ubiquitin-proteasome system (UPS)[11,12] (Figure 1a). Based on our work in this area,[12] we hypothesized that a small hybrid molecule designed to bring mHtt and E3 into close proximity would induce degradation of mHtt in living cells. Since no small-molecular ligand for Htt has yet been discovered, we focused on the aggregation-prone nature of mHtt and planned to utilize small-molecule probes for targeting the protein aggregates (Figure 1b). For this purpose, we focused on BTA (**4**) and PDB (**5**) (Figure S1), because of reports on brain penetration[13,14] and a linker introduction.[15,16] It is not clear whether these probes can specifically bind to soluble oligomers as well as insoluble polyQ aggregates, although Thioflavin T (ThT) (**6**, Figure S1) can bind to polyQ aggregates and soluble oligomers of amyloid beta.[17] As a cIAP1-recognizing moiety, we decided to conjugate bestatin (**7**, Figure S1) through an amide bond, because bestatin methyl amide BE04 (**8**, Figure S1) is more selective for cIAP1 over peptidases as compared with bestatin methyl ester MeBS (**9**, Figure S1), and bestatin amide derivatives do not induce auto-ubiquitination of cIAP1 at low concentration, unlike bestatin ester derivatives.[18] Hence, we designed and synthesized **1** and **2** consisting of cIAP1 ligand BE04 conjugated to BTA or PDB, respectively (Figure 1c and Schemes S1 and S2).

We examined the effects of **1** and **2** on mHtt levels in two fibroblasts derived from two patients with HD (HD fb). At the concentration of 10 μM, **1** and **2** reduced the mHtt level in both HD fb (Figures 2 and S2a), which suggests that **1** and **2** work effectively as down-regulators of mHtt. Compound **1** reduced the mHtt level in a dose dependent manner (Figure S2b)

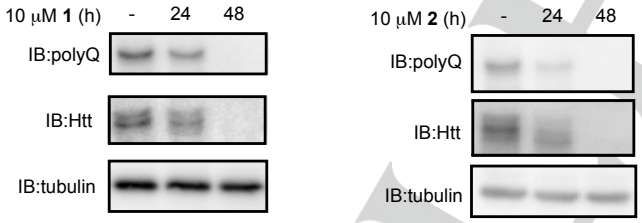

Figure 2. Compounds **1** and **2** reduced the Htt level in HD fb (GM04281). Western blot analyses stained with both anti-polyQ antibody and anti-Htt antibody were performed.

Although these fibroblast cells express both mHtt and wild type Htt (wtHtt), we could hardly detect wtHtt with anti-Htt antibody after treatment with either **1** or **2** (Figures 2 and S2). Therefore, we investigated the activity of the compounds by using fibroblasts derived from a normal subject (normal fb) expressing only wtHtt. First, we confirmed that **1** and **2** reduced the wtHtt level in normal fb (Figure S3a). Next, to confirm that **1** and **2** do not affect transcription of the *HTT* gene itself, we analyzed the mRNA abundance of Htt in HD fb. qPCR analysis showed that **1** and **2** did not affect mRNA abundance (Figure S3b). Taken together, these results indicate that the transcription of *HTT* gene is not targeted by **1** and **2**, but they reduce the level of wtHtt as well as that of mHtt after transcription without cytotoxicity (Figure S4). Considering reports that (1) purified wtHtt containing 32Q formed small aggregates *in vitro*,[19] (2) the smallest polyQ peptide able to bind to ThT (**6**) is 6Q peptide,[20] and (3) ThT can bind to soluble oligomers of amyloid beta as well,[17] we assume that wtHtt also aggregate slightly or form soluble oligomers and **1** and **2** may also interact with small aggregates or soluble oligomers of wtHtt to induce their degradation.

In vitro competitive binding assay[21] by using aggregates of 62Q peptides[22] indicates that **1** and **2** bind to turbid 62Q aggregates (Figure S5). Next, we explored whether **1** and **2** induce non-physiological complex formation [aggregate-compound-cIAP1] by means of two ELISA assays using immobilized model insoluble aggregates of 62Q and GST-fused baculovirus IAP repeat 3 (GST-BIR3),[12a] the bestatin analog-binding site of cIAP1, and immobilized GST-BIR3 and soluble GST-62Q. Small molecules induced interaction between cIAP1 and both insoluble 62Q aggregates and soluble GST-62Q that was blocked in the presence of BTA or MeBS (Figures 3, S6 and S7). It is known that GST-62Q has an amyloid-like conformation,[3] and this result indicates that both small molecules can induce interaction even between soluble oligomers of mHtt and cIAP1.

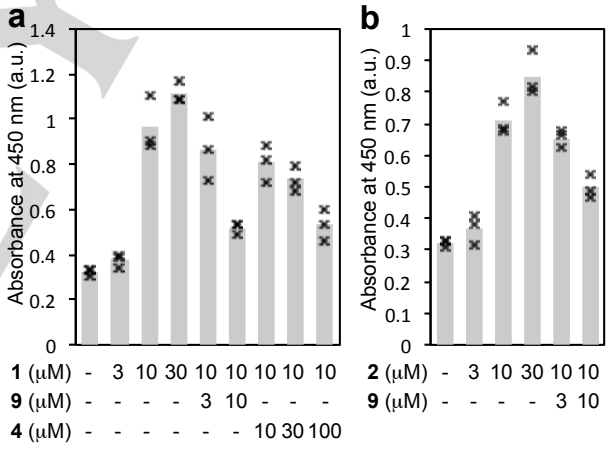

Figure 3. Demonstration of (**a**) **1**-induced and (**b**) **2**-induced interaction between immobilized 62Q aggregates and GST-BIR3 by ELISA. Experiments were performed in triplicate. Gray bars indicate means of each data points, and black cross marks indicate data points.

The hydroxy and amino groups of BE04 are crucial for binding to cIAP1[18] and a derivative not containing the hydroxy and amino groups showed no degradation-inducing activity towards the target protein.[12b] Thus, we designed and synthesized **3** (Figure 1) as a negative control. Neither BTA alone, BE04 alone, the combination of them, nor **3** reduced the mHtt level in HD fb (Figure S8a). Next, the proteasome dependence of the degradation was investigated. Pretreating HD fb with proteasome inhibitors MG132 and bortezomib abrogated the **1**-mediated down-regulation of mHtt (Figure S8b). All these results support our conclusion that **1** and **2** bind to both mHtt

aggregates and cIAP1, and thereby induce proteasomal degradation of mHtt.

The longer the polyQ repeat structure in mHtt, the earlier symptoms appear in patients and the more severe they become,[23] and clinical treatment of HD with longer polyQ is considered to be more difficult. Hence, we investigated whether **1** could induce degradation of mHtt with a much longer polyQ repeat, using the enhanced green fluorescent protein-tagged exon 1 of Htt containing 145Q (145QHtt-EGFP).[24] Compound **1** reduced the 145QHtt-EGFP level in Hela cells transiently expressing 145QHtt-EGFP, but not the EGFP level in Hela cells transiently expressing EGFP (Figure 4a). These hybrid molecules did not decrease the levels of EGFP and tubulin and did not affect cell viability (Figure S4), suggesting high selectivity for Htt over unrelated proteins. In accordance with the results in Figure S3a, the level of 23QHtt-EGFP (this repeat number does not cause HD) was also reduced in Hela cells transiently expressing 23QHtt-EGFP (Figure S9a). In contrast, neither BTA alone, BE04 alone, the combination of them, nor **3** reduced the 145QHtt-EGFP level (Figure S9b). Moreover, to exclude the possibility of accelerated formation of insoluble aggregates of mHtt, fluorescence-microscopy analysis was also performed. Treatment with **1** significantly reduced the number of aggregates of 145QHtt-EGFP (Figures 4b and S10). These results suggest that **1** and **2** can be effective in reducing mHtt with much longer polyQ repeats, and does not enhance the aggregation.

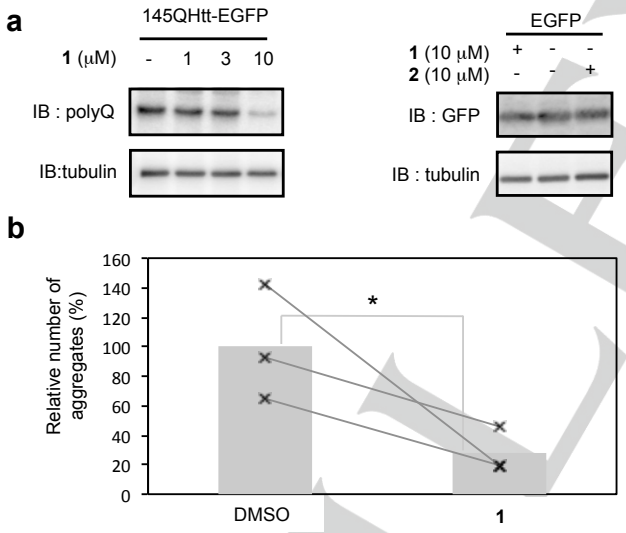

Figure 4. Compound **1** reduces the level of mHtt with an extended polyQ tract and the number of its aggregates. (**a**) Compound **1** dose-dependently reduces 145QHtt-EGFP in Hela cells transiently expressing 145QHtt-EGFP after 18 h treatment, but not the EGFP level in Hela cells transiently expressing EGFP. (**b**) Compound **1** significantly reduces the number of 145QHtt-EGFP aggregates in Hela cells transiently expressing 145QHtt-EGFP after 18 h treatment. Relative number of aggregates was compared using an unpaired, two-sided Student's *t*-test. The *P*-value is indicated by an asterisk (* means $P<0.05$). Gray bars represent means of each independent experiment (n=3), and black cross marks indicate data points.

In summary, we have developed two chemical classes of small hybrid molecules consisting of a cIAP1 ligand linked to a probe for protein aggregates, and we showed that both compounds induced degradation of mHtt in living cells. In particular, they were effective against mHtt in cells derived from HD patients and mHtt with a much longer polyQ repeat sequence (145Q). Analysis of the mode of action revealed that **1** and **2** induce complex formation between Htt aggregates and cIAP1, and this leads to proteasomal degradation of mHtt. Several groups have reported impairment of UPS in neurodegenerative disorders, whereas there is increasing evidence that UPS is still operative in neurodegenerative disorders.[25] We showed that hijacking E3 ligase induced by the small molecule would be effective therapeutic approach for neurodegenerative diseases. We also showed the novel methodology for hybrid small molecule-mediated posttranslational knockdown of target proteins for which small-molecular ligands for aggregation-prone proteins have not been identified. The design concept of targeting the aggregated state of proteins differs from previous approaches of posttranslational knockdown using specific ligands to target proteins. We believe that this methodology would be applicable to target other aggregation-prone proteins, besides Htt, that cause neurodegenerative diseases including AD, PD and other polyQ diseases.

Acknowledgements

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Keywords: Drug design, Huntington's disease, Medicinal chemistry, Neurodegenerative disorders, Small molecule-protein degrader

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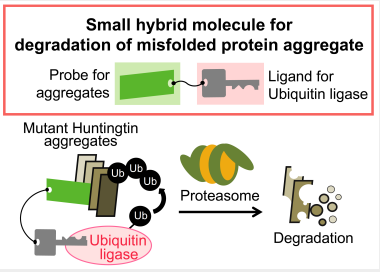

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