PROTACs and Other Chemical Protein Degradation Technologies for the Treatment of Neurodegenerative Disorders

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Recent Progress in PROTACs and Other Chemical Protein Degradation Technologies for the Treatment of Neurodegenerative Disorders

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Abstract: Neurodegenerative disorders (NDs) are a group of diseases that cause neural cell damage, leading to motility and/or cognitive dysfunctions. One of the causative agents is misfolded protein aggregates, which are considered as undruggable in terms of conventional tools, such as inhibitors and agonists/antagonists. Indeed, there is currently no FDA-approved drug for the causal treatment of NDs. However, emerging technologies for chemical protein degradation are opening up the possibility of selective elimination of target proteins through physiological protein degradation machineries, which do not depend on the functions of the target proteins. Here, we review recent efforts towards the treatment of NDs using chemical protein degradation technologies, and we briefly discuss the challenges and prospects.

1. Introduction

1.1. Neurodegenerative Disorders and Treatment Approaches

Neurodegenerative disorders (NDs) are a series of diseases characterized by progressive impairments in motility and/or cognitive function, leading in some cases to death.[1] Alzheimer's disease (AD) is the most common cause of dementia: 10-30% of people >65 years of age are estimated to live with AD.[2] Onset of the major NDs, such as AD, Parkinson's disease (PD), and polyglutamine diseases (polyQDs), is associated with the accumulation of aggregation-prone misfolded proteins (amyloid β (Aβ), tau, α-synuclein, and proteins with abnormally expanded polyglutamine repeats, respectively, in the above diseases).[3] These misfolded proteins accumulate as insoluble fibrillar aggregates via soluble oligomeric intermediates, which are currently considered as the real villain in the pathogenesis[4] (Figure 1). Note that the misfolded proteins often show unusual protein-protein interactions (PPIs) independently of their intrinsic functions. The unusual PPIs cause dysfunctions in specified compartment including nucleus and mitochondria and lead to neuronal cell death.[5] Therefore, the conventional drug discovery program with modification of the intrinsic functions of pathogenic proteins is not suitable for the treatment of NDs.

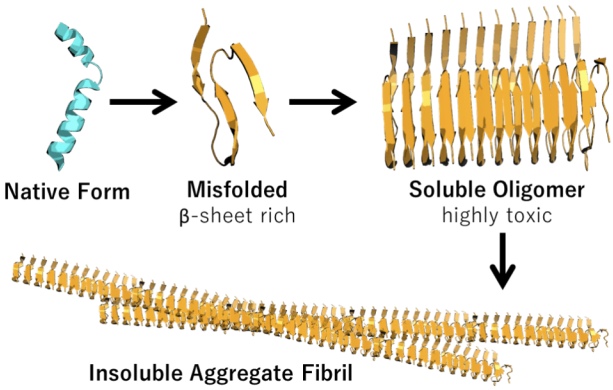


Figure 1. Schematic illustration of the process of misfolded protein aggregation.

Many attempts have been made to develop NDs treatments, generally by employing chemical or biological techniques to eliminate the toxic oligomeric species from neuronal cells. Medicinal chemistry studies have yielded various small molecules that modulate aggregation pathways. Early aggregation modulators were aromatic planar molecules that inhibit aggregate formation by interfering with the interaction of the planar β-sheet surfaces of misfolded proteins to disrupt their stacking.[6] On the other hand, in 2012, an aggregation enhancer was discovered that reduces the population of oligomeric species and increases that of fibrillar aggregates.[7] But, despite this long-established strategy, only one aggregation modulator is currently in clinical trial.[8] Gene silencing techniques, such as RNA interference (RNAi), antisense oligonucleotides, and genome editing, have also attracted attention.[9] Indeed, some in vivo applications for NDs have already been reported, exploiting adeno-associated virus (AAV) or non-viral delivery systems, and clinical trials for amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD, one of the nine polyQDs) are ongoing.[10] Nevertheless, delivery is still problematic, because non-viral delivery systems are invasive and less

effective, while viral delivery systems pose safety issues. The possibilities of off-target effects and interference with endogenous RNAi pathways are also concerns.[11] Passive immunization therapy is another approach to reduce misfolded proteins, albeit it works extracellularly. Several antibodies for Aβ are currently evaluated in phase III clinical studies.[12] Biogen and Eisai announced one of the Phase III study of their aducanumab, an monoclonal antibody against Aβ, met its primary endpoint in 2019, and they are now preparing to submit Biologics License Application to the FDA.[13] However, passive immunization is costly and shows poor BBB permeability (typically ~0.1% of the injected antibody cross BBB).[12] Indeed, aducanumab requires high-dose administration.

1.2. Emerging Prospect: Chemical Protein Degradation

Today, chemical inhibitors, agonist/antagonists, and ion channel openers/blockers are widely used for various diseases. On the other hand, the misfolded proteins in NDs are generally considered as 'undruggable' in that, for example, they lack ligand-binding sites that could be targets for inhibitors or modulators, and the neuronal cell death is induced independently of the intrinsic functions of them. Thus, novel therapeutic strategies are required. One such strategy is to lower the levels of target proteins by using small molecules or peptides to promote their degradation.[14–17] The chemical protein degradation strategy aims to direct eukaryotic protein degradation machineries, including the ubiquitin-proteasome system (UPS; for the details, see section 1.3) or autophagy (for the details of autophagy, see section 3), towards a protein of interest (POI) by modulating the relevant protein-protein interactions. The concept of hybrid molecules with a dual mode of action provided a clue to the development of the chemical protein degradation technologies;[18] one of the technologies this purpose

developed for is UPS induction using hybrid molecules 'Proteolysis Targeting Chimeras (PROTACs).[19]

1.3. Development of PROTACs

In UPS, a ubiquitin ligase (E3) repeatedly labels its protein substrate with ubiquitins, which are activated by a ubiquitin-conjugating enzyme (E2), to form poly-ubiquitin chain on lysine residues of the protein substrate. Subsequently, a large protease complex proteasome recognizes the ubiquitin chain and hydrolyzes the substrate.[20]

In 2001, Crews and co-workers pioneered the development of PROTACs[21] which are hetero-bifunctional molecules comprised of a ligand for an E3 linked to a ligand for the POI. These hybrid molecules serve to bring the POI and the E3 into close proximity and enable the POI to be ubiquitinated even though it is not an endogenous substrate of the E3, thereby leading to proteasomal degradation (Figure 2).

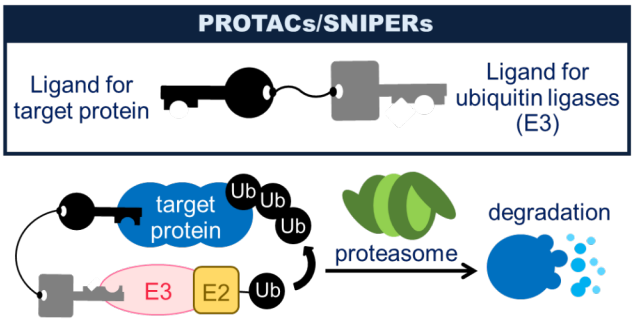


Figure 2. The concept of PROTACs-mediated protein degradation.

The first-generation PROTACs are peptide-based molecules that employ β-TrCP or von Hippel-Lindau (VHL) recognizing peptides as E3 ligands. However, their cell-permeability is problematic and these PROTACs require micro injection or

incorporation of cell-penetrating peptide (CPP) sequences for use in living cells.[21,22] To address these problems, Crews et al. developed a small hybrid molecule consisting of a ligand for androgen receptor (AR) and nutlin-3, a small-molecular murine double minute 2 (MDM2, an E3) inhibitor.[23] This hybrid molecule has been described as the first small-molecular PROTAC, but this may not be strictly accurate,[24] because AR is actually an endogenous substrate of MDM2.[25] In addition, nutlin-3 itself enhances MDM2-mediated AR degradation.[26]. Taking account of these questions, our group focused on the induction of non-physiological protein degradation by small molecules, and in 2010 we reported cell-permeable, small-molecular PROTACs (also known as SNIPERs: Specific Non-IAP-dependent Protein Erasers) which recruit inhibitor of apoptosis protein (IAP) family members possessing E3 activity.[27] We subsequently applied IAP-mediated protein degradation to various proteins located in cytosol, nucleus, cell membrane, and mitochondria.[28,29] In 2015, the Crews group and the Bradner group independently developed VHL- and cereblon (CRBN)-based small-molecular PROTACs, respectively.[30,31] These PROTACs were the first to achieve potent degradation of the POI with DC_{50} values of nanomolar order in cells. These achievements dramatically accelerated the advance of the technology,[32] like a "Cambrian explosion," and led to multiple applications, including HaloTag-fused proteins,[33,34] bromodomain-containing proteins,[35–37] kinases,[38–40] and phosphodiesterase,[41] as well as in vivo studies.[30,31,42] Further exploration of E3 for PROTACs is attractive, because only a limited number of E3s has been utilized so far.[43] Besides IAP, VHL and CRBN, five E3s have been exploited for small-molecular PROTACs to date;[44–48] however, this corresponds to only a few percent of E3s. Mechanistic studies have shown the unique aspects of PROTAC technology. For

example, studies using promiscuous warhead as a ligand for POI revealed that accessibility to ternary complex formation involves in their selectivity, suggesting that "PROTACization" of promiscuous drugs might be an idea to improve their selectivity.[49] Since late 2019, optical control of PROTACs have attracted attention and more than five papers were published so far.[50]

In the past few years, PROTACs technology has attracted commercial interest, with the major focus being on PROTACs for cancer therapy.[51,52] Our group reported double degradation of IAP and an oncogenic protein by a hybrid molecule employing an IAPs pan antagonist in 2012,[53] suggesting that double protein degradation of oncogenic IAPs and oncogenic proteins is a promising approach for cancer treatment. We believe that this feature affords a major advantage over other PROTACs that utilize the ubiquitin ligases VHL and CRBN. In 2019, a similar approach targeting oncogenic proteins with PROTACs employing MDM2 inhibitors resulted in synergistic activity.[44,54] The structural insights into ternary complex (POI-PROTAC-E3)[55] reported in 2017 have facilitated rational molecular design of PROTACs for cancer therapy. Two PROTACs from Arvinas, Inc. entered phase I clinical studies for certain cancers in 2019.[56]

2. Proteolysis-Targeting Chimeras for NDs therapy

2.1. Peptide PROTACs Aimed at AD Therapy

In 2016, the group led by Chen and Li reported a tau-targeting PROTAC with potential for AD treatment; this was the first attempt to apply PROTACs to the

treatment of NDs.[57] They designed the tau-targeting all-peptide PROTAC **1** (Figure 3); this is a 32-a.a. peptide consisting of, from the N-terminus, a motif for tau recognition, a linker peptide, a motif for VHL recognition, and D-Arg_8 as the CPP. They successfully demonstrated **1**-mediated degradation of tau through UPS in cell cultures and in vivo. Notably, **1** also ameliorated the neurotoxicity of Aβ.

Another peptide PROTAC for AD was developed by Jiang, You and colleagues.[58] It is noteworthy that they harnessed CRL^{Keap1} by using a 9-a.a. peptide sequence for Keap1 recognition, which was identified by the same group.[59] Their peptide PROTAC **2** (Figure 3) induced UPS-mediated degradation of tau protein in cell lines.

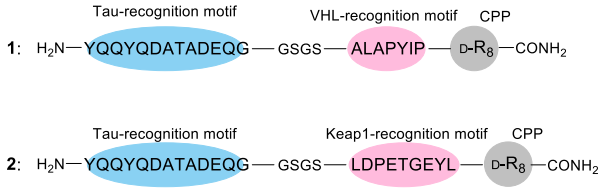


Figure 3. Structures of tau-targeting peptide-based PROTACs **1** and **2**.

2.2. Small-Molecular PROTACs for NDs therapy

The greatest obstacle to developing small-molecular PROTACs for NDs is that no selective small-molecular ligand for NDs-related proteins has yet been discovered. To address this problem, we exploited small-molecular binders to misfolded protein aggregates, and developed compounds **3** and **4** (Figure 4) as the first all-small-molecular PROTACs targeting mutant huntingtin (mHtt, an aggregate-prone neurotoxic protein involved in HD) in 2017.[60,61] In the design of **3** and **4**, we used benzothiazoles BTA and PDB, which are PET tracers for misfolded protein aggregates, as aggregate binders,

and linked them to ligands for IAP (therefore these compounds can be categorized as SNIPERs). Compounds **3** and **4** successfully induced a UPS-mediated decrease of mHtt in primary cells from HD patients, as well as in HeLa cells transfected with mHtt exon-1 bearing a long polyQ repeat. In brief, i) mechanistic analysis established that **3** did not decrease *HTT* mRNA, ii) an artificial complex between IAP and aggregates was detected by means of ELISA iii) a negative control compound without affinity for IAP did not reduce the mHtt level, and iv) involvement of proteasomal degradation of mHtt was confirmed by co-treatment with a proteasome inhibitor. Furthermore, **3** also decreased the amount of mHtt aggregates in cells. We observed the degradation of wild-type Htt as well, but not that of green fluorescent protein (GFP) as a control, and we concluded that wild-type Htt also forms small oligomers that can be recognized by aggregate binders, leading to PROTAC-mediated degradation. Targeting protein aggregates seems to be a promising strategy to develop PROTACs targeting pan-misfolded proteins. Indeed, we found that compounds **3** and **4** also reduce the levels of mutant ataxin-3, mutant ataxin-7 and mutant atrophin-1, which are misfolded proteins implicated in other polyQDs.[62] In 2019, Gray and Haggarty's group reported, independently of our group, another small-molecular PROTAC **5** targeting protein aggregates (Figure 4).[63] Their compound contains the core 5*H*-pyrido[4,3-*b*]indole scaffold of T807, a PET tracer for tau aggregates, as an aggregate binder, and this is linked to pomalidomide for recruitment of CRL^{CRBN}. They demonstrated that **5** triggers UPS-mediated tau clearance in neurons derived from patients with frontotemporal dementia (FTD), as well as promoting recovery of FTD neurons from tau-mediated stress vulnerability.

Based on the abstract of a scientific meeting, Arvinas, Inc. appears to have discovered small-molecular PROTACs that potently degrade pathologic tau species in tauopathy mice.[64] Notably, these PROTACs can be administered peripherally and cross the BBB to show activity.

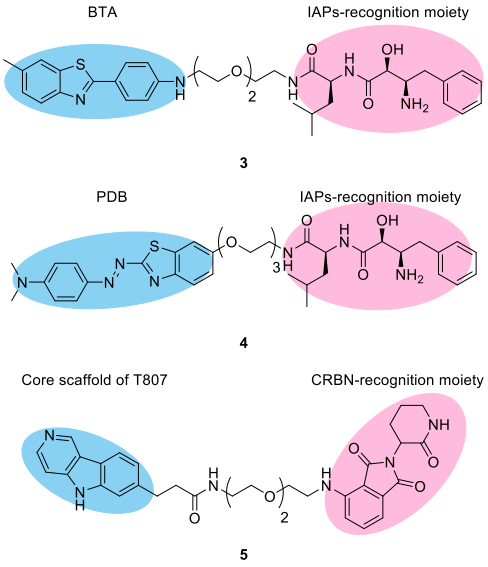


Figure 4. Structures of misfolded protein-targeting small-molecular PROTACs **3**, **4** and **5**.

3. Targeted Autophagy Inducers

Autophagy is defined as the delivery of cytoplasmic cargo to lysosomes for degradation. At present, autophagy can be divided, by mode of cargo delivery, into the following three categories (Figure 5): macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA).[65]

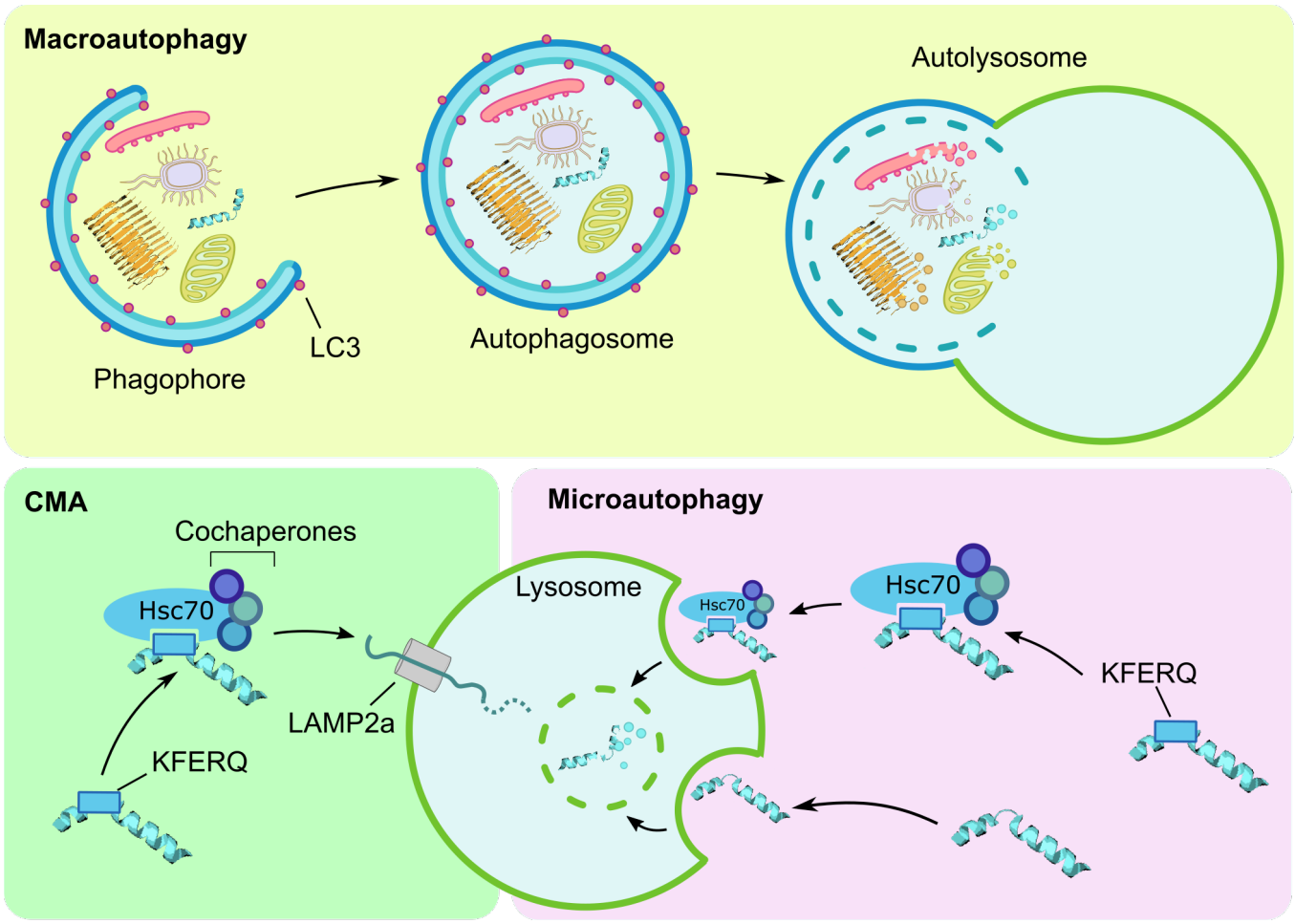


Figure 5. Schematic illustration of autophagic pathways in mammals.

As neurons are terminally differentiated cells, they cannot dilute cytoplasmic materials by cell division, and they depend heavily on basal autophagic bulk clearance.[66] Therefore, autophagy is an attractive therapeutic target for NDs treatment.

3.1. Chaperone-Mediated Autophagy-Based Approaches

CMA is a selective autophagic process, in which proteins containing the KFERQ sequence are recognized by chaperone heat shock cognate 70 kDa (Hsc70) and co-chaperones, followed by delivery to and internalization into lysosomes via lysosome-

associated membrane protein type 2a (LAMP2a), leading to lysosomal degradation. Besides the consensus Hsc70 binding motif KFERQ, α-synuclein has the unique Hsc70 binding motif VKKDQ, which is also directed to the CMA machinery (Figure 5).[67] Hence, these two sequences can be utilized as CMA-targeting warheads. Note that Hsc70 also involves in microautophagy but LAMP2a does not. The following two reports have proven the involvement of LAMP2a.

In 2010, Nukina and co-workers reported targeted protein degradation by hijacking the CMA machinery for selective clearance of mHtt.[68] The authors designed a DNA construct coding a 46-a.a. peptide **6** (Figure 6), consisting of KFERQ and VKKDQ sequences linked to two copies of polyglutamine binding peptide 1 (QBP1). This reduced mHtt in mouse brain, ameliorated motor dysfunction, and improved survival ratio without decreasing the body weight of wild-type mice. Additionally, **6** conjugated to monomeric red fluorescent protein (mRFP) as a reporter can decrease accumulation and aggregation of other polyQ proteins, such as mutant ataxin-3 and mutant AR, suggesting the potential of this approach for pan-polyQDs treatment. As for synthetic peptides, Wang's group developed a cell-permeable peptide **7** as a CMA inducer targeting α-synuclein.[69] Compound **7** is a 35-a.a. peptide consisting of, from the N-terminus, a TAT sequence from HIV as a CPP, an α-synuclein-binding motif, and CMA-targeting motifs (Figure 6). The α-synuclein-binding motif they employed is a 10-a.a. stretch from β-synuclein that is known to interact with α-synuclein (K_d = 1 μM, fluorescence polarization).[70] The authors demonstrated that the addition of synthetic **7** to the culture medium of primary neurons successfully reduced the level of wild-type α-synuclein, as well as A53T mutant, a cause of familial PD, in a lysosome-dependent manner.

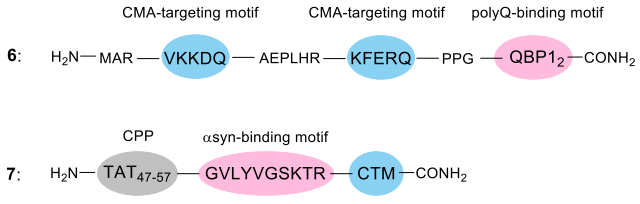


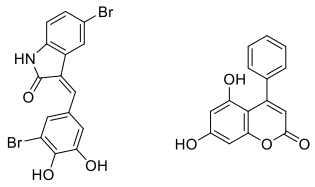
Figure 6. Structures of CMA inducers **6** and **7**. CTM: CMA-targeting motifs.

3.2. Macroautophagy-Based Approach

Macroautophagy is the best-characterized machinery of autophagy, in which the cytoplasmic cargoes are sequestered in autophagosomes, which are double-membrane vesicles formed by elongation of the phagophore, a cup-shaped membrane. The fusion of an autophagosome with endosome or lysosome leads to degradation of the cargo (Figure 5).[71] Microtubule-associated proteins light chain 3B (LC3B), attached to the inner membrane surface of the phagophore, acts as a receptor for macroautophagy, for encapsulation of the cargo.[72]

In 2019, Li et al. performed a small-molecule microarray (SMM)-based screening to identify compounds that tether mHtt and LC3B together to encapsulate mHtt in autophagosomes.[73] Excluding hits in the screening with wtHtt, the authors identified small molecules **8** and **9** (Figure 7) that induce allele-selective clearance of mHtt in an autophagy-dependent manner. Further, all the compounds they identified successfully rescued HD-relevant phenotypes in vivo, e.g. resulting in prolongation of lifespan in *Drosophila* HD models and amelioration of motor dysfunction in mouse HD models. It is noteworthy that the identified compounds are not hetero-bifunctional molecules but molecular glues, suggesting that the SMM-based screening could be used to identify BBB-permeable compounds with the same activity. Further structure-activity

relationship studies of the compounds identified in the report should uncover the core structure for binding to LC3B without perturbation of its activity.



8 **9**

Figure 7. Structures of molecular glues **8** and **9** that bring mHtt and LC3B together.

4. Hydrophobic Tagging

Eukaryotic cells operate a protein quality control system to remove misfolded proteins and their aggregates. Key players in this process are molecular chaperones called heat-shock proteins (HSPs) that refold the misfolded proteins or facilitate their degradation through UPS or autophagy.[74] HSPs recognize misfolded proteins through their exposed hydrophobic residues, which are buried inside proteins in their native folding. This system can be utilized to degrade POIs. For example, fulvestrant is composed of an endogenous ER ligand and a hydrophobic alkylsulfinyl group as a HSPs recruiting moiety; this enables it to degrade ER, and it is clinically used to treat ER-positive metastatic breast carcinomas. The Crews group has generalized this hydrophobic tagging technique by designing small molecules, termed hydrophobic tags (HyTs), consisting of a hydrophobic adamantyl group linked to a ligand for a POI to induce HSP-mediated protein degradation.[75,76]

Li and his co-workers have reported two series of HyTs aimed at the treatment of AD (in 2017) and ALS (in 2019), employing tau- and TDP-43-targeting peptides,

respectively, as warheads for the POI.[77,78] Compound **10**, the tau-targeting HyT shown in Figure 8, reduced tau in living cells in a proteasome-dependent manner. Further, the intravenous administration of this HyT successfully degraded tau in brains of AD model mice, suggesting that **10** is BBB-permeable. As for the TDP-43-targeting HyT, the authors designed a repertoire of peptides consisting of adamantane(s), linker peptides, TDP-43-targeting peptides, and CPPs. Among those peptides, **11**, containing two adamantyl groups as hydrophobic groups, was the most effective, reducing the TDP-43 levels in living cells as well as in TDP-43-overexpressing *Drosophila* models. But, although these HyTs showed degradation activity in vivo, high doses (20-150 μM in the cell) were required, and slight cytotoxicity was observed.

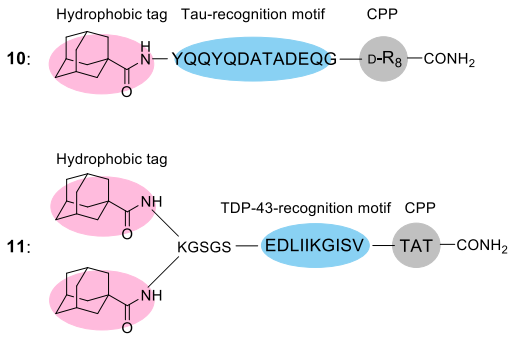


Figure 8. Structures of HyTs **10** and **11**.

5. Summary and Outlook

Drug discovery for NDs faces at least two problems: (1) aggregation-prone proteins cause diseases independently of their intrinsic functions, and (2) drugs that can cross the BBB are required. Point 1 means that conventional drug discovery strategies, which rely on the modulation of functions of proteins, e.g., with inhibitors or agonists/antagonists,

are not appropriate. Point 2 means that gene silencing of pathologic proteins is problematic due to the difficulty of delivering nucleic acids into the patient's brain, in addition to safety concerns. Chemical protein degradation has already overcome the point 1 because this approach eliminates pathologic proteins by utilizing common structural features of misfolded proteins, i.e., β-sheet-rich structure, but not the inherent function or structure of the pathogenic protein. The phase III result of aducanumab, which decreases extracellular Aβ aggregates, also encourages chemical protein degradation approaches towards ND therapy. Aducanumab are close to clinical use but this approach has yet to solve point 2. In fact, it requires high-dose administration (aducanumab required 10 mg/kg) and occurs brain swelling (edema) in a dose-dependent manner.[79] On the other hand, small-molecular protein degraders decrease intracellular aggregation-prone proteins such as tau and polyQ proteins, and might solve point 2. Although few BBB-permeable chemical degraders have been reported so far, improvement of their bioavailability should be more feasible than antibodies. Their hetero-bifunctional structure results in a fairly high molecular weight (MW), which is unfavorable for bioavailability due to violations of 'Lipinski's rule of 5 (Ro5)'.[80] However, the number of orally-available agents which are out of Ro5 (so-called 'beyond Ro5' drugs) has recently been increasing. Notably, an orally-active small-molecular PROTAC was also reported in 2019.[81] Analysis of these successes has been run by several groups and is expected to offer principles to design bioavailable chemical protein degraders. Moreover, to lower their MW, click-formed PROTACs (CLIPTACs) technology should be suitable. CLIPTAC is a technique to form PROTACs in cells through bio-orthogonal click reactions of the warheads for E3 and POI; this technique should make it possible to assemble PROTAC molecules in situ in the brain from the

two distinct bioavailable small molecular warheads administered separately.[82] Furthermore, use of HyT or discovery of druglike E3 ligands also holds a key for a good bioavailability. HyT possessing less hydrogen bonding acceptors/donors and lower MW might be suitable for a central nervous system (CNS) drug, but specificity by HyT technology should be carefully examined. Molecular glue-type protein degraders are likely ideal for CNS drugs from the perspective of Ro5 although rational design and identification of the molecules are difficult.

A potential issue for the protein degradation strategy is that the protein degradation machineries might be impaired in NDs, although this remains controversial: some reports suggest that misfolded protein aggregates inhibit UPS and autophagy, though opposite results have also been reported.[83,84] Therefore chemical protein degradation approaches for NDs will need to be evaluated carefully. Even so, the overexpression of proteins implicated in UPS or autophagy pathway was reported to reduce misfolded protein aggregates,[83,85] so the impairment, if it exists, may be overcome by artificial enhancement of the efficacy of the protein degradation systems.

Each chemical protein degradation technology described above is currently not ideal; they have advantages and drawbacks as summarized in Table 1. Of these chemical degraders, as of writing this minireview, molecular glues and small molecular PROTACs are likely to be promising approaches in the view of potency and CNS druglikeness. However, the premature judgment should not be made because these technologies still have been evolving. For example, autophagy-targeting chimeras (AUTACs), a novel small-molecular technology inducing autophagy, were reported in 2019, which is a breakthrough towards the rational design of small molecular autophagy inducers.[86] Other emerging approaches to the treatment of NDs are also feasible. For

instance, in 2020, Nakatani and co-workers reported a novel polyQDs therapeutic strategy based on the use of a small-molecular binder to the hairpin structure of abnormally expanded CAG-repeat DNA (coding polyQ).[87] This small molecule prevented further expansion of the CAG repeat and instead induced its contractions, resulting in the decrease in misfolded protein aggregates in cells and in mice. Existing and prospective technologies decreasing the levels of aggregation prone proteins seem to offer considerable potential for the treatment of NDs in the future.

Table 1. Summary of advantages and drawbacks of chemical protein degradation technologies described in this minireview.

Technologies	Advantages	Drawbacks
PROTACs	Relatively potent activity (0.1-10 μM) Well-examined technology	Violations of rule of 5
CMA inducers	Misfolded protein aggregates are physiological substrates of autophagy	Poly-peptides Weak activity
Molecular glues	Small molecules with low MW Potent activity (10-75 nM)	Weak degradation efficacy (up to 50%) Rare lead identification/optimization
HyTs	CNS druglike property of the tag moiety	Poly-peptides; weak activity (100 μM) Low specificity

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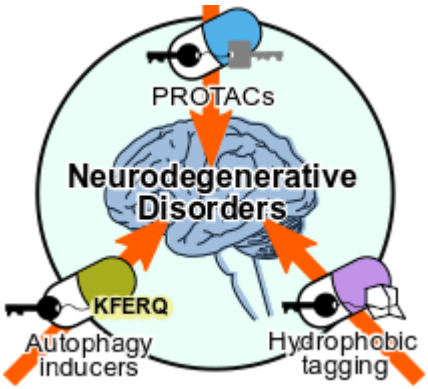

Conventional drug discovery approaches are difficult to apply for the removal of the misfolded protein aggregates that are thought to cause neurodegenerative disorders (NDs). In contrast, new chemical protein degradation technologies such as PROTACs seem promising for NDs therapy, opening up the possibility of selective elimination of 'undruggable' target proteins by utilizing physiological protein degradation machineries. Here, we review recent progress and prospects.

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