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Research paper

Discovery of a Keap1-dependent peptide PROTAC to knockdown Tau by ubiquitination-proteasome degradation pathway

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ABSTRACT

Induced protein degradation by PROTACs has emerged as a promising strategy to target nonenzymatic proteins inside the cell. The aim of this study was to identify Keap1, a substrate adaptor protein for ubiquitin E3 ligase involved in oxidative stress regulation, as a novel candidate for PROTACs that can be applied in the degradation of the nonenzymatic protein Tau. A peptide PROTAC by recruiting Keap1-Cul3 ubiquitin E3 ligase was developed and applied in the degradation of intracellular Tau. Peptide **1** showed strong in vitro binding with Keap1 and Tau. With proper cell permeability, peptide **1** was found to colocalize with cellular Keap1 and resulted in the coimmunoprecipitation of Tau and Keap1. The results of flow cytometry and western blotting assays showed that peptide **1** can downregulate the intracellular Tau level in both time- and concentration-dependent manner. The application of Keap1 siRNA silencing and the proteasome inhibitor MG132 confirmed that peptide **1** could promote the Keap1-dependent poly-ubiquitination and proteasome-dependent degradation of Tau. The results suggested that using PROTACs to recruit Keap1 to induce the degradation of Tau may show promising character in the treatment of neurodegenerative disease. Besides, our research demonstrated that Keap1 should be a promising E3 ligase adaptor to be used in the design of novel PROTACs.

1. Introduction

Targeting non-enzymatic proteins inside the cell is the high hanging fruit in drug discovery [1]. Recently, induced protein degradation has been an emerging strategy to modulate intracellular concentrations of 'undruggable' proteins, including scaffolding proteins, transcription factors and other non-enzymatic proteins inside the cell [2–6]. Compared with nucleic acid-based agents that reduce the production of a particular protein [7], induced protein degradation shows promising characters in drug discovery paradigm [8,9]. More recently, Crews and others have developed bifunctional molecules, including proteolysis-targeting chimaeras (PROTACs) and hydrophobic tagging (HyT), as the platform to induce protein degradation by utilizing cellular protein quality control mechanisms. HyT is to tag small hydrophobic molecules to the target protein, which gives a mimic of misfolded protein to be easily degraded in vivo [10]. PROTACs utilize heterobifunctional molecules that harness the ubiquitin–proteasome system (UPS) by recruiting an E3 ligase to a protein of interest (POI), leading to proximity-induced ubiquitylation and subsequent degradation of the protein [2]. Therefore, PROTACs show more promising characters than HyT in the drug discovery process. The binding selectivity of the protein ligand and the substrate specificity of E3 ubiquitin ligases together confirmed the selectivity of PROTACs [11]. Additionally, PROTACs are event-driven, which can cycle through multiple rounds of activity, removing substoichiometric quantities of proteins [12]. Besides, PROTACs can be easily developed by linking identified protein binders to the appropriate E3 ligase ligands once plentiful E3 ligase ligands are available. Since the discovery of β-transducin repeat-containing

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protein (βTRCP) [13], only a few E3 ligases including mouse double minute 2 homolog (MDM2) [14], inhibitor of apoptosis proteins (IAPs) [15,16], cereblon (CRBN) [17,18] and von Hippel–Lindau (VHL) [19–21] have been identified as appropriate candidates for the design of PROTACs. This field remains underdeveloped.

Kelch-like ECH-associated protein-1 (Keap1) functions as a substrate adaptor protein for a Cullin 3 (Cul3)/Ring-Box1 (Rbx1)-dependent E3 ubiquitin ligase complex [22]. The most well-known substrate of the Keap1-Cul3 ubiquitin E3 ligase complex is the transcription factor NF-E2-related factor-2 (Nrf2), which is the master regulator of oxidative stress. Keap1-dependent ubiquitination of Nrf2 can be finely tuned according to the cellular redox state. Dysregulation of the Keap1-Nrf2 signaling has been proven to be closely related with the oxidative stress and inflammatory-related diseases [23]. Thus, it is quite interesting to explore whether Keap1 is a potential target hijacked by PROTACs to induce protein degradation.

Aberrant protein aggregation is the most prominent feature of neurodegenerative diseases [24]. These aggregates, like many non-enzymatic proteins involved in diseases, are built up of intrinsically disordered proteins with no active pockets, which can hardly be regulated by traditional occupancy-driven drugs [25,26]. PROTACs provide a possible way to selectively remove these protein aggregates. Microtubule associated protein Tau aggregation is a typical character in neurodegenerative diseases, and Tau degradation represents a promising strategy for treating neurodegenerative diseases [27,28]. Besides, oxidative stress, coupling with oxidative injury and inflammatory conditions, is another important pathogenic factor for neurodegenerative diseases [29,30]. Several studies have indicated the therapeutic effects of Keap1-Nrf2 pathway in many chronic neurodegenerative diseases [31–33]. Thus, PROTACs hijacking Keap1, an inhibitor of Nrf2, to downregulate Tau could be a promising strategy in the treatment of neurodegenerative diseases. In this study, we planned to design PROTACs that recruit Tau to the Keap1-Cul3 ubiquitin E3 ligase complex for ubiquitination and subsequent proteasome-mediated degradation (Fig. 1).

2. Results and discussion

2.1. Design and synthesis of a peptide PROTAC hijacking Keap1

A PROTAC molecule has two functional parts, one binds to the POI while the other binds to an E3 ligase. In order to explore the E3 ligase adaptor Keap1 in protein degradation, we designed a peptide PROTAC that consisted of a moiety for recognizing Tau joined to a moiety for binding to Keap1 (Fig. 2). Our recent work has established one peptide, Ac-LDPETGEYL-OH, as a strong binder of Keap1

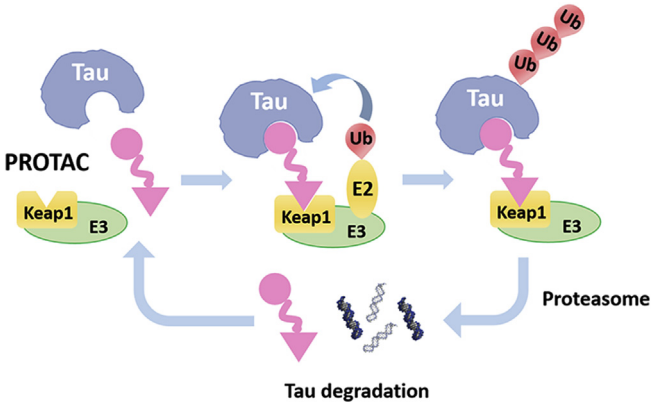

Fig. 1. Mechanism of PROTACs technology with Keap1 and Tau.

Fig. 2. Design of the peptide PROTAC hijacking Keap1.

[34]. It has been identified to be the most potent short linear sequence for Keap1. Thus, this nine-residue sequence was chosen as the Keap1 recognition domain (Table 1). To achieve selective recognition of Tau, a peptide from β-tubulin that interacted with Tau, YQQYQDATADEQG, was chosen as the moiety to recognize Tau as previously reported [35]. In order to increase the flexibility of the peptide, a short peptide, GSGS, was chosen to tether the Tau recognition moiety to the Keap1 binding moiety [36]. Finally, a cell-penetrating peptide, poly-D-arginine (RRRRRRRR) [37], was fused to the C terminus of the peptide to enhance uptake into the cell and resist nonspecific proteolysis [38], which resulted in the sequence of peptide **1** (Fig. 2). It was synthesized using a standard solid phase peptide synthesis approach with Fmoc chemistry for further study.

2.2. Peptide 1 can interact with Keap1 and Tau

PROTAC-mediated formation of a ternary complex that consists of E3 ligase and POI is the basis of protein ubiquitination. Thus, the high binding strength of both PROTAC-E3 ligase and PROTAC-POI is indispensable for induced protein degradation. Firstly, the isothermal titration calorimetry (ITC) assay was carried out to assess whether peptide **1** retained its binding affinity to Keap1 [39,40] and Tau. As shown in Fig. 3, peptide **1** retained its strong binding to Keap1 with a K_d value of 22.8 nM and to the target protein Tau with a K_d value of 763 nM, suggesting that peptide **1** can induce the formation of a ternary complex comprising E3 ligase and POI.

2.3. Peptide 1 can enter cells and promote the cellular interaction between Keap1 and Tau

To characterize the ability of peptide **1** to penetrate cells, a TAMRA-Peptide **1** (Peptide **1** labeled by carboxytetramethylrhodamine [TAMRA] at N-terminal) was synthesized. The wild-type SH-SY5Y human bone marrow cancer cells were incubated with 10 μM TAMRA-peptide **1** for different time periods (0, 2, 4, 8, 12, 24 h). The flow cytometry assay was employed here to detect the intracellular TAMRA fluorescence intensity. As shown in Fig. 4A and B, the fluorescence intensity increased gradually during the incubation time and stabilized after 12 h. Confocal microscopy was achieved on the wide-type SH-SY5Y cells to visualize the intracellular distribution of peptide **1**. The results further confirmed that peptide **1** could enter cells and colocalize with Keap1 (Fig. 4C). In order to investigate whether cellular Keap1 interacted with Tau in a peptide **1**-dependent manner, we performed a Co-IP assay in the wild-type SH-SY5Y cells. Cells were incubated with two concentrations of peptide **1** (10, 20 μM) followed by immunoprecipitation. The results showed that Tau can coimmunoprecipitate with Keap1 (Fig. 4D). Conversely, immunoprecipitation of Keap1 also brought down the endogenous Tau (Fig. 4E). The stable in vitro binding of peptide **1** to Keap1 and the target protein Tau, together with proper cell permeability ensured peptide **1** could be a potent tool in further investigation.

2.4. Peptide 1 can induce Tau degradation in Tau over-expressed cell lines

To evaluate the induced Tau degradation effects of the peptide