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TDP-43 specific reduction induced by Di-hydrophobic tags conjugated peptides

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ABSTRACT

TAR DNA binding protein 43 (TDP-43) is a key target in amyotrophic lateral sclerosis (ALS) treatment. Here, based on hydrophobic tagging strategy, we designed and synthesized a series of single or double hydrophobic tags conjugated peptides D1-D8. Among them, it was found that D4 displayed strongest ability to induce TDP-43 degradation in cells. D4 could reduce TDP-43 induced cytotoxicity. Besides, D4 could reduce TDP-43 levels in a transgenic *drosophila* model.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a presently incurable neurodegenerative disease. Its clinical manifestations include limbs and trunk muscle atrophy, severe motor dysfunction or even respiratory failure. Currently, no effective treatments are available, so it is essential to develop novel therapeutic strategies [1].

In 2006, nuclear protein TAR DNA binding protein 43 (TDP-43) was identified, which is the key pathological protein for ALS [2]. Studies showed that wild-type TDP-43 overexpression in mammalian cells or transgenic animal models could cause TDP-43 deposits, which led to cytotoxicity and progressive motor weakness [3–6]. In addition, inclusions of TDP-43, the pathological hallmark, were observed in nearly all ALS patients [2].

Besides, growing evidence suggested that dysfunction in the intracellular protein quality control systems and protein homeostasis network could be involved in the pathogenesis of ALS [7–10]. it was also found that the concentration of TDP-43 monomeric pathogenic protein has a great influence on its aggregation process. The higher concentration of TDP-43 monomer, the faster speed of its aggregation [6]. Therefore, imbalance of TDP-43 levels could be a common feature in TDP-43-induced pathogenesis, and reducing TDP-43 levels may be a potential method for therapeutic intervention.

TDP-43 is flexible and its structure showed intrinsic disorder [11]. The methods to reduce the level of undruggable proteins usually include genetic and chemical strategies [12–15]. Although genetic method to regulate protein levels is efficient, there are some disadvantages such as safety challenges [12–13]. Generally, chemical methods could regulate protein levels through activating the cellular protein quality control system [14]. In recent years, some chemical methods have attracted much attention, such as hydrophobic tagging method [16–19], the auxin-inducible degron (AID) [20], phthalimide conjugations [21–22] and proteolysis targeting chimeras (PROTACs) [23–26]. In addition, these methods have been widely used to induce the degradation of some disease-related proteins [27–30].

It was reported that protein attached with a hydrophobic group could mimic the state of the denatured protein and consequently be degraded by the proteasome [15].Crews and his coworkers firstly developed small-molecules to directly mimic the protein unfolding state. This method was known as hydrophobic tagging method (HyT) [18]. These small-molecules could bind to a bacterial dehalogenase (the HaloTag protein) and increase its hydrophobicity. The small-molecule with the adamantyl group could lead to degradation of many Halo Tag fusion proteins such as luciferase, $HRas1^{G12V}$, GFP, ROR2 [18,31–32]. Furthermore, an adamantane moiety coupled to the ligand of target protein was found to effectively induce the degradation of some important disease-related proteins (the androgen receptor and the pseudokinase Her 3) [33–35].

The hydrophobic tagging method was effective in robustly and specifically degrading protein of interest, which was also proved useful

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Table 1
Sequence of the synthesized peptides.

Entity	Appended Tag	Number of Adamantane	Linker	Recognize TDP-43	Cell-penetrating peptide
D1	—	1	GSGS	EDLIIKGISV	RRRRRRRR[a]
D2	—	2	KGSGS	EDLIIKGISV	RRRRRRRR
D3	—	1	GSGS	EDLIIKGISV	GRKKRRQRRR[b]
D4	—	2	KGSGS	EDLIIKGISV	GRKKRRQRRR
D5	—	1	GSGS	MNFGAFSINP	RRRRRRRR
D6	—	2	KGSGS	MNFGAFSINP	RRRRRRRR
D7	—	1	GSGS	MNFGAFSINP	GRKKRRQRRR
D8	—	2	KGSGS	MNFGAFSINP	GRKKRRQRRR
CP1	—	—	GSGS	EDLIIKGISV	RRRRRRRR
CP2	—	—	GSGS	EDLIIKGISV	GRKKRRQRRR
CF-D4	CF	2	KGSGS	EDLIIKGISV	GRKKRRQRRR

CF: 5(6)-carboxyfluorescein; a: Poly-D-Arginine; b: TAT.

to manipulate disease-relevant proteins. Thus, we intend to apply HyT method to regulate TDP-43 levels.

2. Results and discussion

2.1. Design and synthesis of a series of multifunctional peptides

We designed a series of multifunctional peptides, which contained three parts

a hydrophobic motif, a TDP-43 recognition motif and a cell-penetrating peptide (Table 1, Fig. 1). To facilitate peptides penetrating into cells, TAT (RRRQRRKKRG) [36] and Poly-D-Arginine $(D\text{-}Arg)_8$ [25] were chosen as the motif of cell-penetrating peptide. To bind to TDP-43 specifically, two peptides sequences (MNFGAFSINP and EDLIIKGISV) [37], TDP-43 core aggregation domains, were chosen as TDP-43 recognition motif. Simultaneously, a different number of adamantly groups (single and double) were selected as hydrophobic moieties. In addition, linkers (GSGS and KGSGS) were applied to increase these multifunctional peptides' flexibility [38]. We expected that these multifunctional peptides could get into cells quickly, lead to TDP-43 degradation specially *in vitro* and *in vivo*.

Through pre-screening, it was found that D5-D8 were difficult to use for further investigation due to their extremely low solubility. The reason is probably that the sequence MNFGAFSINP has high hydrophobicity. Thereby, we had to give up these four peptides.

2.2. D3 and D4 displayed a relatively low cytotoxicity

To test the cell survival of D1-D4, the mouse neuroblastoma N2a cells were treated with different concentration of D1-D4. Through MTT experiments, we found that D1 and D2 were highly toxic, so the concentration was small during the experiment

D3 and D4 were less toxic, so the concentration was large during the experiment (Fig. 2A-D).

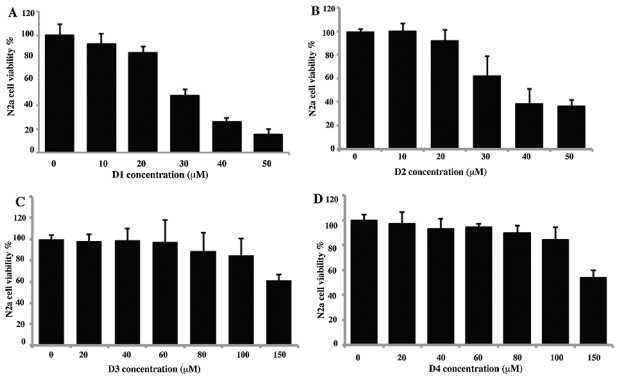

Fig. 2. D3 and D4 has a relatively low cytotoxicity. (A) Cell viability results of the wild type N2a cells with the treatment of 0 μM, 10 μM 20 μM, 30 μM, 40 μM and 50 μM D1 respectively. (B) Cell viability results of the wild type N2a cells with the treatment of 0 μM, 10 μM 20 μM, 30 μM, 40 μM and 50 μM D2 respectively. (C) Cell viability results of the wild type N2a cells with the treatment of 0 μM, 20 μM 40 μM, 60 μM, 80 μM, 100 μM, 150 μM D3 respectively. (D) Cell viability results of the wild type N2a cells with the treatment of 0 μM, 20 μM 40 μM, 60 μM, 80 μM, 100 μM, 150 μM D4 respectively. Data are detected by MTT assay. Vales are means ± SD, n = 5.

2.3. D4 showed strongest degradation ability on TDP-43 protein

To investigate the degradation ability of D1, D2, D3 and D4, we firstly generated a stable 162–414 CTDP-43-EGFP A315T (CTDP-43) overexpressed cell line, which is based on the mouse neuroblastoma N2a cell line (Fig. S1). Then we incubated the overexpressed cells respectively with 20 μM D1, 20 μM D2 100 μM D3 and 100 μM D4 for 24 h. Finally, we used flow cytometry to detect EGFP fluorescence intensity changes and western blot to assess CTDP-43 level. The flow cytometry results indicated that D4 could cause the maximum reduction in fluorescence intensity

the other three peptides presented comparatively weak ability to reduce fluorescence intensity (Fig. 3A). The western blot results demonstrated the same conclusion as the flow cytometry (Fig. 3B, Fig. S2). Therefore, D4 was chosen for further verification.

2.4. D4 could penetrate into cells within a short time

To characterize whether D4 could get into cells, we firstly synthesized CF-D4. Appending a fluorescence tag (5(6)-carboxyfluorescein) to the N-terminal of D4 led to CF-D4. Then the N2a cells were treated with 100 μM CF-D4 with 1 h, 2 h, 4 h, 8 h and 12 h respectively. The flow

Fig. 1. Design of a series of multifunctional peptides that can induce TDP-43 degradation.

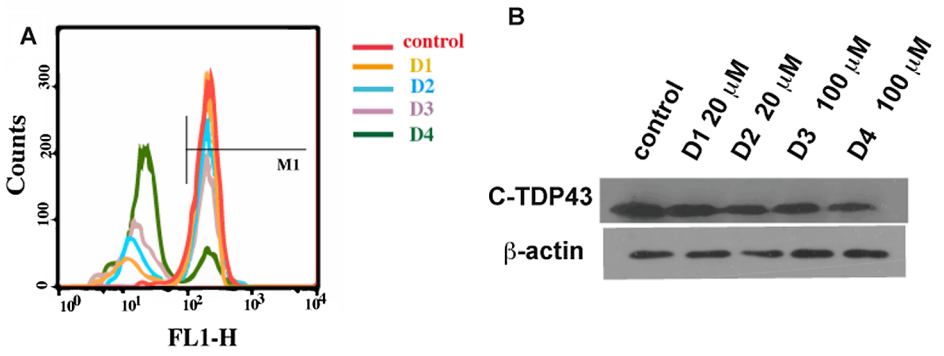

Fig. 3. D4 showed strongest degradation ability on CTDP-43 protein. (A) The flow cytometry results of the CTDP-43 overexpressed cells treating with vector, 20 μM D1, 20 μM D2, 100 μM D3 and 100 μM D4 for 24 h. The gray horizontal line represents the fluorescence positive signal threshold. (B) The western blot results of the overexpressed cells treating with vector, 20 μM D1, 20 μM D2, 100 μM D3 and 100 μM D4 respectively for 24 h. Anti-TDP-43 (C-terminal) antibody (Sigma) was used to detect CTDP-43 (162–414) and anti-β-actin antibody (sigma) was used to detect β-actin.

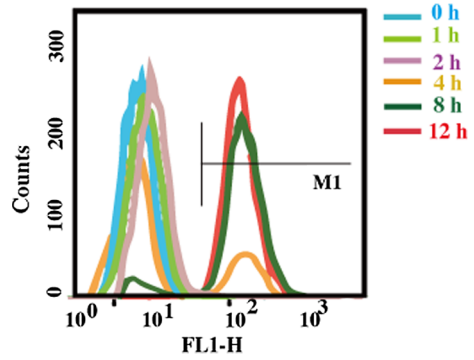

Fig. 4. D4 could penetrate into cells within a short time. The flow cytometry results of the mouse N2a neuroblastoma cells treating with 100 μM CF-D4 for 1 h, 2 h, 4 h, 8 h and 12 h respectively. The gray horizontal line represents the fluorescence positive signal threshold.

cytometry results showed that intracellular fluorescence intensity is gradually increasing as the incubation time becomes longer. After 4 h, a slight enhancement of fluorescence intensity can be seen. Moreover, after 8 h, a distinct enhancement of fluorescence intensity can be observed.

These indicated that D4 could penetrate into cells within a short time (Fig. 4).

2.5. D4 could induce TDP-43 degradation in a dose- and time- dependent manner.

To further evaluate D4 degradation ability, the CTDP-43 overexpressed cells were incubated with increasing concentrations of D4 for 24 h or with 100 μM D4 for different time. The flow cytometry and western blot assay were applied again, the results of which demonstrated that D4 could significantly induce the degradation of CTDP-43 in a dose- and time- dependent manner (Fig. 5A-5F and Fig. S3). We also synthesized CP1 and CP2 (detailed sequence was seen in Table 1) that both didn't contain the hydrophobic motif as controls. The CTDP-43 overexpressed cells were treated with vector, 100 μM CP1, 100 μM CP2 and 100 μM D4 respectively. The western blot results indicated that CP1 and CP2 couldn't lead to degradation of CTDP-43 (Fig. 5G, Fig. S5). Thereby, it was proved that the hydrophobic motif was essential for degradation.

2.6. D4 could reduce the CTDP-43-induced cytotoxicity

To verify whether CTDP-43 reduction could rescue CTDP-43-induced cytotoxicity, we incubated the wild type N2a cells and the TDP-43 overexpressed cells with vector and increasing concentrations of D4 respectively. The MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) results showed that

D4 could significantly reduce CTDP-43-induced cytotoxicity after treating the CTDP-43 overexpressed cells with 100 μM D4 (Fig. 6).

2.7. D4 could reduce the TDP-43 level in TDP-43 overexpressed Drosophila

To verify whether D4 could induce TDP-43 degradation *in vivo*, we have constructed a full-length TDP-43 overexpressed *Drosophila* model by using MHC-Gal4 to drive the TDP43 expression in fly indirect flight muscles. D4 was mixed with 200 μL liquid *Drosophila* food and The final concentration of D4 in *Drosophila* food was 200 μM. The TDP43 flies were fed with the food for two weeks. Then Take 10 drosophilae and take their thoracic sections, use NP40 extract (containing PI) to pulverize them. Applying western blot to detect the level changes of TDP-43 in *Drosophila*. An obviously reduction of TDP-43 level in treated *Drosophila* can be seen, which demonstrated that D4 could reduce the level of TDP-43 in *Drosophila* (Fig. 7, Fig. S6).

3. Conclusion

In this work, we designed and synthesized a series of multi-functional peptides to induce the degradation of TDP-43. Among them, D4 showed the strongest ability in inducing CTDP-43 degradation. It was demonstrated that D4 could enter into cells within a short time and have a relatively low cytotoxicity. Moreover, D4 could induce TDP-43 degradation *in vitro* and *in vivo*. Applying hydrophobic tags to induce TDP-43 degradation may become an effective potential strategy for ALS treatment.

4. Methods

4.1. Peptides synthesis and RP-HPLC purification

We used the standard solid phase peptide synthesis method to synthesize all the peptides as mentioned in the papers. Materials in the coupling steps were Rink Amide-AM resin, 4-equiv Nα-Fmoc-protected amino acids, 4-equiv activator 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 4-equiv activator 1-Hydroxy-7-azabenzotriazole (HOAT) and 8.0-equiv activator base N, N-Diisopropylethylamine (DIEA). Time for each amino acid coupling was 45 min. 20% piperidine in N,N-Dimethylformamide (DMF) was used to cleave Fmoc groups. (TFA/water/phenol/thioanisole/1, 2-Ethanethiol = 82.5/5/5/5/2.5) was used as the cleavage reagent. The cleavage reagent was used to cleave the peptides from resin for 3 h. After that, TFA was removed and the peptides were precipitated by diethyl ether. Then peptides were collected by centrifugation with 8000 rpm for 10 min. Centrifugation should be done at least three times. Finally, the peptides were analyzed and purified by HPLC. ESI/MS was used to identify the peptides. The hydrophobic tag used in this paper was 1-adamantane carboxylic acid that was conjugated to the N terminus of the peptide by the peptide coupling method. We coupled 5(6)-carboxyfluorescein (CF) to the N terminus of D4 to get CF-D4 by

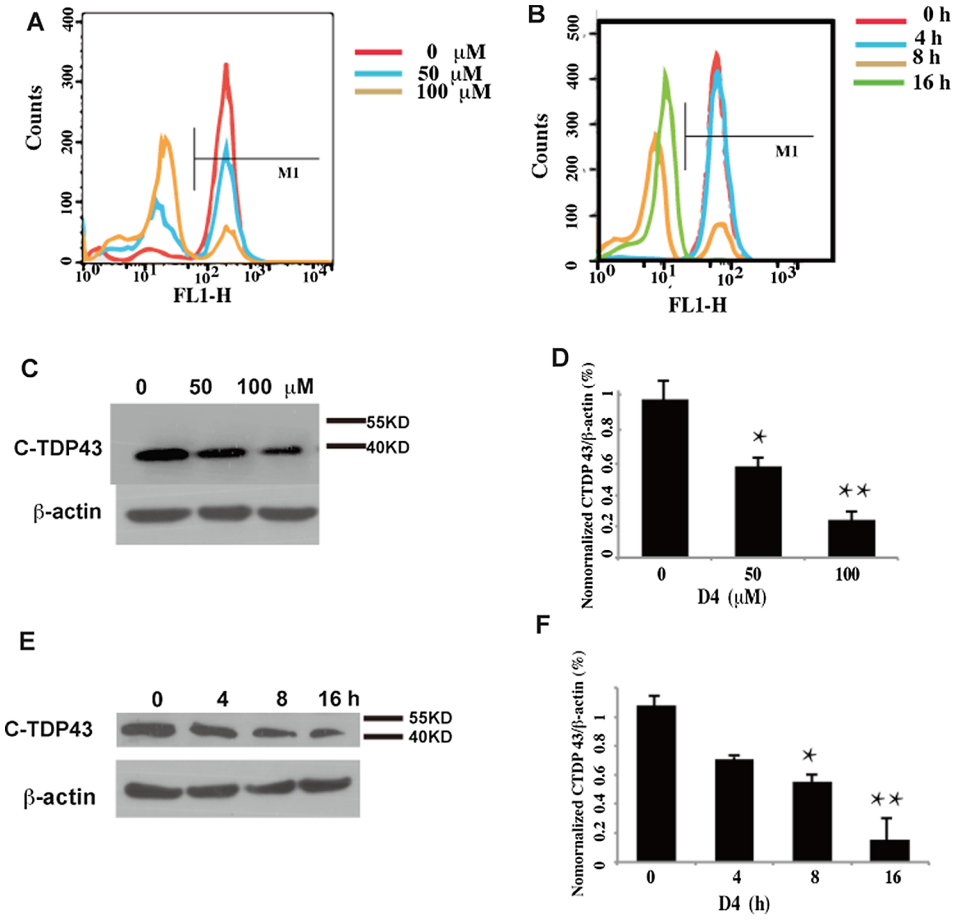

Fig. 5. D4 could induce CTDP-43 degradation in a dose- and time- dependent manner. (A) The flow cytometry results of the CTDP-43 overexpressed cells treated with 0 μM, 50 μM and 100 μM D4 for 24 h respectively. The gray horizontal line represents the fluorescence positive signal threshold. (B) The flow cytometry results of the CTDP-43 overexpressed cells with the treatment of 100 μM D4 for 0 h, 4 h, 8 h and 16 h respectively. The gray horizontal line represents the fluorescence positive signal threshold. (C) Western blot results of the CTDP-43 overexpressed cells with the treatment of 0 μM, 50 μM and 100 μM D4 for 24 h respectively. (D) a statistically significant result of Fig. 5C. The level of TDP-43 is measured by densitometry and normalized with β-actin level. Statistical significance compared to the control (0 μM) was analyzed by student's *t*-test, *P < 0.05. **P < 0.01. ***P < 0.001. (E) Western blot results of the CTDP-43 overexpressed cells with the treatment of 100 μM D4 for 0 h, 4 h, 8 h and 16 h respectively. (F) a statistically significant result of Fig. 5E. The level of TDP-43 is measured by densitometry and normalized with β-actin level. Statistical significance compared to the control (0 h) was analyzed by student's *t*-test, *P < 0.05. **P < 0.01. ***P < 0.001. (G) Western blot results of the CTDP-43 overexpressed cells with the treatment of 100 μM vector, 100 μM CP1, 100 μM CP2 and 100 μM D4 for 24 h respectively. Anti-TDP-43 (C-terminal) antibody (Sigma) was used to detect CTDP-43 and anti-β-actin antibody (sigma) was used to detect β-actin. Full blot images for 5C and 5E are shown in Fig. S4.

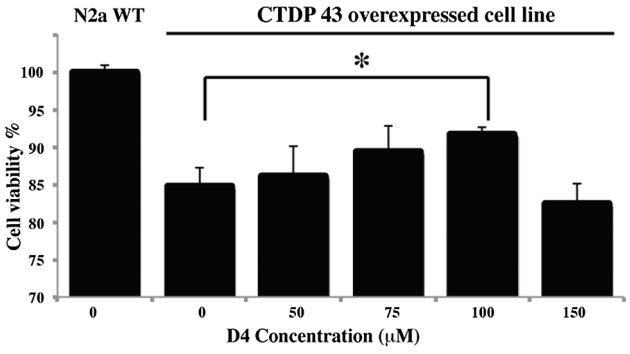

Fig. 6. D4 could reduce the CTDP-43-induced cytotoxicity. Cell viability results of the wide type N2a cells and CTDP-43 overexpressed N2a cells in the same number of cells. CTDP-43 overexpressed N2a cells with the treatment of 0 μM, 50 μM 75 μM, 100 μM and 150 μM D4 respectively. Data are detected by MTT assay. Vales are means ± SD, n = 5. Statistical significance was analyzed by student's *t*-test, *P < 0.05. **P < 0.01.

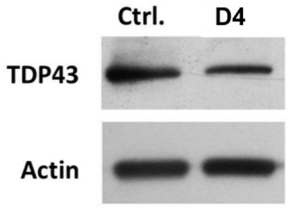

Fig. 7. D4 could reduce the TDP-43 level in TDP-43 overexpressed *Drosophila*. Western blot results of the protein extracts from TDP-43 flies fed with 200 μM D4 for two weeks. Anti-TD-*P*43 (full length) antibody (Sigma) was used to detect TDP-43 and anti-β-actin antibody (sigma) was used to detect β-actin, β-actin was used as the loading control.

using the same way as indicated above. A preparative C18 column of reverse phase (5 μM, 20 × 250 nm; flow 6.0 mL/min, YMC, Japan; solution A: 100% water +0.06% TFA, solution B; 80% acetonitrile + 20% water + 0.06% TFA) was used to purify the peptides on a SHIMADZU LC-6AD. The pure peptides that we obtained were lyophilized and stored at -20 °C.

4.2. Cell culture and gene transduction

The mouse N2a neuroblastoma cells and CTDP-43 (162–414)-EGFP A315T overexpressed cells were cultured in 45% Dulbec-co's Modified Eagle's Medium (DMEM), 45% Minimum Essential Medium (α-MEM) supplemented with Fetal Bovine Serum (FBS) and Streptomycin (100 μg/mL) and Penicillin (100 μg/mL). Cells were cultured in a high-temperature sterilization CO2 incubator (Thermo 371). Culture environment was maintained at 37 °C, 5% CO2. To construct a stable CTDP-43 (162–414) A315T overexpressed cell line, the mouse N2a neuroblastoma cells were treat with Lipofectamine™2000 (Invitrogen) and the plasmid of pEGFP-N3 CTDP-43 (162–414) A315T. Lipofectamine™2000 could transfer the plasmid of pEGFP-N3 CTDP-43 (162–414) A315T into cells. Finally, using 800 μg/mL antibiotic G418 to screening cells as to get a stable cell line.

4.3. Western blotting assay

Firstly, the CTDP-43 (162–414) overexpressed cells were treated with cycloheximide (CHX, 10 μg/mL) for 2 h. Then the cells were incubated with drugs in different time or different concentrations as experiments designed. After that, the cells were collected and lysed with P0013 lysis buffer (Beyotime) including 1% cocktail protease inhibitors (Calibiochem). BCA kit (Beyotime) was used to quantify protein level. Finally, anti-TDP-43 (C-terminal) antibody (Sigma) or anti-TDP-43 (full length) antibody (Sigma) was used as primary antibody to detect CTDP-43 (162–414) or TDP-43 (full length) respectively. Monocional Anti-β-actin antibody (sigma) was used to detect actin. Goat anti-Rabbit IgG H &L (HRP)(sigma) and Rabbit anti-Mouse IgG H&L (HRP)(sigma) were used as the secondary antibody. Every experiment was independently done at three times.

4.4. Flow cytometry assay

The CTDP-43 (162–414) overexpressed cells were incubated with D1-D4 in different time or different concentrations as experiments designed, after treating with cycloheximide (CHX) (10 μg/mL) for 2 h. Then the cells were washed and suspended by PBS buffer (pH = 7.4). Lastly, the FACS Calibur flow cytometer (BD Biosciences) was applied to detect the changes of fluorescence intensity. The number of cells observed was 10,000. Every experiment was independently done at three times.

4.5. MTT assay

The mouse N2a neuroblastoma cells were incubated with increasing concentrations of D1-D4 as experiments designed with a density of 2×10^5/mL in a 96-well plate for 24 h. Then each well was added 20 μL, 5 mg/mL MTT reagent for 4 h. After removal of medium, each well was added 150 μL dimethyl sulfoxide (DMSO). At last, a Biotek Synergy 4 microplate reader was used to detect the absorbance value of 490 nm. Each well was repeated for five times.

4.6. Construction of Drosophila model and drosophila experiments

Firstly, we constructed a plasmid containing pUAST-hTDP43. Then the plasmids were injected into *Drosophila* embryos so that a TDP-43 transgenic Drosophila strain was constructed. Finally, The Drosophila UAS-hTDP43 flies were crossed with MHC-Gal4 to obtain the progeny MHC-Gal4 > UAS-TDP-43 flies (abbreviated as TDP43 flies). D4 was mixed with 200 μL of liquid *Drosophila* food, the final concentration of D4 was 200 μM, and it was dropped on filter paper. The drosophila was fed for 2 weeks. And the food was changed every day.

4.7. Detection of TDP-43 levels in vivo by western blotting assay

Take 10 drosophilae and take their thoracic sections, use NP40 extract (containing PI) to pulverize them. The TDP-43 level was tested by the ordinary western method. Anti-TDP-43 (full length) antibody (Sigma) was used to detect TDP-43 and anti-β-actin antibody (sigma) was used to detect actin.

5. Ethical conduct of animal experiments

All animals applied in this work were treated in compliance with the Institutional Animal Care and Use Committee (IACUC) and the Laboratory Animal Facility of Tsinghua University.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Author contributions

Na Gao did most experiments, analyzed the data, and wrote the paper. Yun-Peng Huang, Ting-Ting Chu and Bing Zhou helped to do the drosophila experiments. Yu-Fen Zhao and Yong-Xiang Chen analyzed the data. Yan-Mei Li designed the experiments, analyzed the data, and wrote the paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.11.042.

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