

Xyloketal-derived small molecules show protective effect by decreasing mutant Huntingtin protein aggregates in *Caenorhabditis elegans* model of Huntington's disease

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Abstract: Huntington's disease is an autosomal-dominant neurodegenerative disorder, with chorea as the most prominent manifestation. The disease is caused by abnormal expansion of CAG codon repeats in the *IT15* gene, which leads to the expression of a glutamine-rich protein named mutant Huntingtin (Htt). Because of its devastating disease burden and lack of valid treatment, development of more effective therapeutics for Huntington's disease is urgently required. Xyloketal B, a natural product from mangrove fungus, has shown protective effects against toxicity in other neurodegenerative disease models such as Parkinson's and Alzheimer's diseases. To identify potential neuroprotective molecules for Huntington's disease, six derivatives of xyloketal B were screened in a *Caenorhabditis elegans* Huntington's disease model; all six compounds showed a protective effect. Molecular docking studies indicated that compound **1** could bind to residues GLN369 and GLN393 of the mutant Htt protein, forming a stable trimeric complex that can prevent the formation of mutant Htt aggregates. Taken together, we conclude that xyloketal derivatives could be novel drug candidates for treating Huntington's disease. Molecular target analysis is a good method to simulate the interaction between proteins and drug compounds. Further, protective candidate drugs could be designed in future using the guidance of molecular docking results.

Keywords: Huntington's disease, mutant Huntingtin, xyloketal derivatives, *Caenorhabditis elegans*, protein misfolding, molecular target

Introduction

Huntington's disease (HD) is a devastating, rare, dominantly inherited brain disorder with typical symptoms of choreatic movements, neuropsychiatric disturbance, and cognitive impairment.¹ It is caused by an elongated polyglutamine (polyQ)-encoding CAG (cytosine-adenine-guanine) repeat in the *IT15* gene.^{2,3} There is still no effective treatment or cure for HD. Pathologically, it is characterized by the presence of protein inclusions containing mutant Huntingtin (mHtt) and progressive neuronal loss in the brain.^{2,4} Mutations, extensions of the polyglutamate portion of the protein, alter the stability and native structure of Htt protein, leaving it prone to aggregation; accumulation of these protein aggregates disrupts normal cellular function, eventually leading to neurodegeneration,⁵ whereas inhibition of protein aggregation has been shown to reduce neuronal death in experimental studies.⁶ Some specific proteins can also interact with mHtt to change the protein structure. For example, the interaction of protein Rhes with mHtt might improve mouse behavior in HD mouse model.⁷ Glyceradehyde-3-phosphate

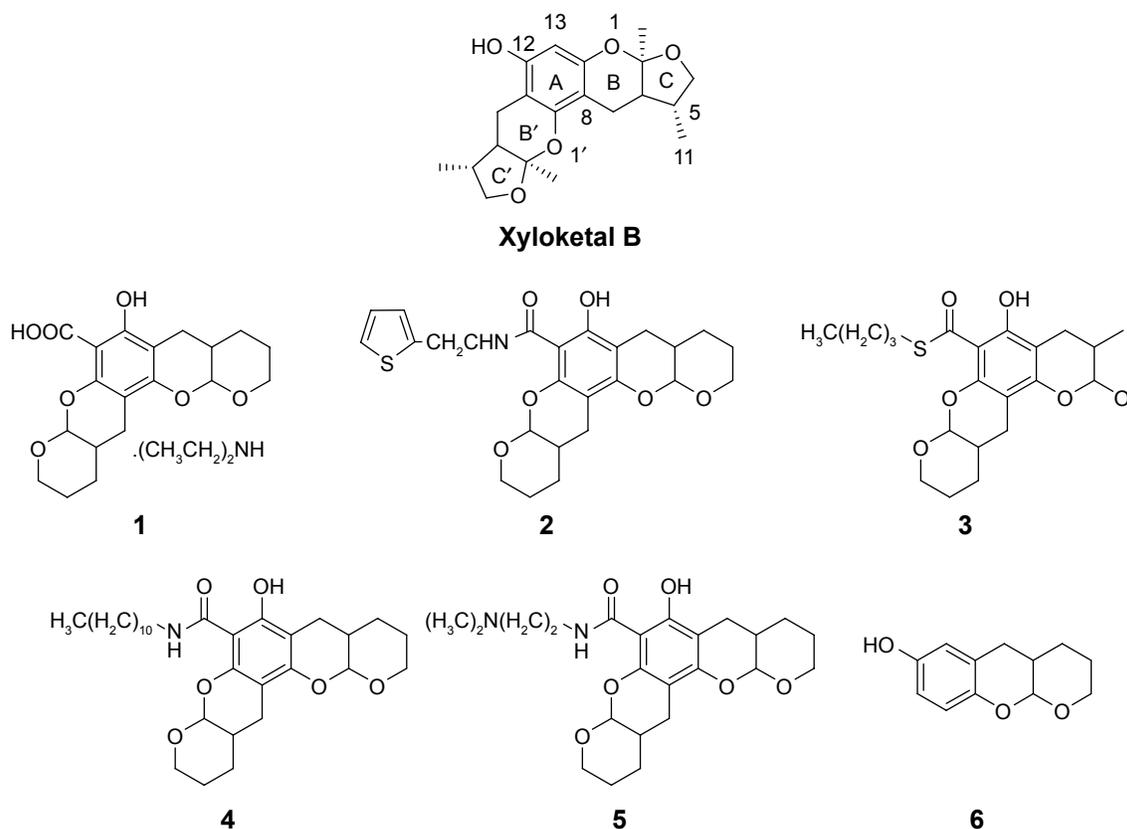


Figure 1 Structure of xyloketal B and compounds 1–6.

dehydrogenase could reduce the aggregation of long polyQ repeats and cytotoxicity in the cell and in a fly model.⁸ Thus, inhibition of pathogenic protein aggregation may have potent clinical applications to ameliorate HD.

The marine environment often provides a sink for the discovery of many novel, natural, and active compounds. Xyloketal B, an extract from marine mangrove fungi,⁹ has exhibited potent neuroprotection in different models of neurological diseases, including Parkinson's disease and epilepsy.^{10–13} Xyloketal B has unique bicyclic acetal moieties fused to its aromatic core structure (Figure 1), which can be easily modified to improve and expand its activity.^{5,14} Given that some xyloketal derivatives have the ability to hydrogen bond, xyloketal may have the potential to bind to mHtt proteins and disrupt the process of Htt aggregation, thereby attenuating the pathological progression of HD.

Caenorhabditis elegans is a small soil nematode that has a very short life span.^{15,16} *C. elegans* has emerged as an ideal model for the study of neurodegenerative diseases because of the high degree of conservation in molecular and cellular pathways between these worms and mammals.¹⁷ Additionally, the transparent body and expression of fluorescent tags such as green fluorescent protein (GFP) in transgenic

C. elegans allow simultaneous investigation of both cellular and behavioral phenotypes. Several *C. elegans* HD models have been developed recently, which recapitulate key features of HD pathogenesis.^{18–21} For example, expression of different-length polyQ variants in *C. elegans* can induce length-dependent aggregation and toxicity.^{22–24}

In the present study, we generated a *C. elegans* model expressing polyQ fused with GFP in muscle cells. We then used the model to examine the anti-mHtt toxin properties of six xyloketal derivatives and investigated the underlying mechanisms of action by conducting molecular docking studies.

Materials and methods

Chemicals and reagents

Xyloketal derivatives 1–6 (Figure 1, compounds 1–6) were synthesized and purified by the School of Chemistry and Chemical Engineering, Sun Yat-sen University, People's Republic of China. The purity of xyloketal B and its derivatives was >99% as examined using high performance liquid chromatography and nuclear magnetic resonance spectroscopy. All compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C until use.

Culture conditions and generation of *C. elegans* model

C. elegans strains N2 (wild type) and CB4088 were purchased from CGC (*Caenorhabditis* Genetic Center, Minneapolis, MN, USA). All worms were handled using standard methods unless otherwise stated.²⁵ They were cultivated on nematode growth medium (NGM) plates in a 20°C incubator and fed on living *Escherichia coli* strain OP50. The *E. coli* OP50 cells were precultured overnight at 37°C after spreading on the surface of NGM plates.

Plasmid construction for expressing polyQ fused with GFP in *C. elegans* muscle cells was performed essentially as described previously.²⁶ The target polyQ fragments were obtained from plasmid pPcDNA3.1/Myc-HisA, which encoded the N-terminal 63 amino acids of the huntingtin gene plus a normal or expanded polyQ repeat (Htt-N63-16Q or Htt-N63-148Q). The polyQ-encoding sequence was then ligated into vector pPD95.86 between the *Xho*I and *Bam*HI sites. GFP was cloned by polymerase chain reaction and inserted into pPD95.86 between sites *Kpn*I and *Sac*I, creating myo-3::Q16-GFP and myo-3::Q148-GFP for expression in the muscle cells.

Transgenic worms were generated by microinjection of engineered plasmid into the gonad of *C. elegans* N2. To allow integration of the polyQ-GFP construct, worms were subjected to UV exposure. They were then mated with CB4088 worms to decrease the UV-induced random damage to the whole genome sequence.²⁷ The HD transgenic line was selected based on fluorescence in muscle cells under optical imaging.

Bioactivity assays

Age-matched worm specimens were used for all bioactivity assays. Adult worms were lysed by hypochlorite treatment to collect synchronized eggs. After 24 hours, the hatched L1 larvae were transferred to fresh NGM plates supplemented with DMSO or the indicated compound, which had been previously dissolved in *E. coli* OP50 and were present on the plates at a final concentration of 100 µM. As the concentration of DMSO was not more than 1 µL per 1,000 µL, the DMSO showed no bioactivity. All plates were cultured in a 20°C incubator until the animals were 4 days old.

In the heat-shock life span assay, the synchronized, treated worms were incubated at 37°C. At least 50 animals were randomly selected after they were treated by each compound. Their viability and mortality rate were counted every hour. Death was defined as a halt of food intake or nonresponsiveness to gentle mechanical touch.²⁸ In bending assays, at least 30 randomly selected animals from each group were

used to examine the body bending frequency. Bending was determined in M9 buffer as a change in the direction of the bend in the midbody until the animal returned to its original position. The number of bends in 20 seconds was counted for each assay.²⁹ All bioactivity assays were repeated at least three times and the mean values were assessed.

Imaging and mHtt aggregate quantification

The number of mHtt aggregates per worm was counted in live animals using confocal microscopy (Leica SP5II) equipped with epifluorescence optics at 20× magnification. The aggregates were defined as discontinuous structures in the form of dots or string-like fibrils. The procedure for worm preparation was the same as for heat-shock/bending assays. Age-synchronized, day 4 worms were loaded onto fresh agar slides which were prepared 2 hours before and treated with 20 µM NaN₃.

Molecular docking

Molecular docking was performed using Surflex-Dock (SYBYL[®]8.1 molecular modeling software Tripos, St. Louis, MO, USA). The crystal structure of Htt36Q3H-EX1-C1 (Protein Data Bank code 4FE8), a trimer of MBP-Htt36Q3H-EX1, was retrieved from the PDB (<http://www.pdb.org/>). As the critical point for understanding mHtt toxicity, the mHtt polyQ region was extracted for molecular docking study.³⁰ Ligands were sketched and minimized using Powell optimization in the presence of the Tripos force field with a convergence criterion of 0.001 kcal/mol Å and then assigned with Gasteiger–Hückel charges. Automatic docking was employed. Other parameters were established by default in the software.

Western blot

In order to detect the level of mHtt expression in worms' lysates, animals weighing ~50 mg (all in L4 stage) were selected from the control group and drug-treated group and washed with water for three times in order to remove the OP50 cells around *C. elegans* body. Cracked the worms in radio immunoprecipitation assay (containing 1 mM phenylmethanesulfonyl fluoride) and treated with low temperature ultrasound pyrolysis processing method. And then, the protein was boiled for 10 minutes in sodium dodecyl sulfate (SDS) loading buffer. After electrophoresis on 8% SDS gel, proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane under semidry conditions and subjected to Western detection. Primary antibody was the polyclonal anti-GFP antibody at a dilution of 1:3,000

(NB600-308; Novus Biologicals, Littleton, CO, USA). At the same time, 1:4,000 dilution of mouse monoclonal anti- β actin was used as control. Secondary antibody was a HRP-linked anti-rabbit IgG antibody at a dilution of 1:5,000 (#7074; Cell Signaling Technology, Beverly, MA, USA). All the antibodies were diluted in TBST/2% milk powder. All the pictures were captured by ChemiDoc Imaging System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Statistics

All the data were analyzed using SPSS Statistics 18 software. Survival data were analyzed by the Kaplan–Meier method. One-way analysis of variance (ANOVA) was followed by a Tukey's post hoc test to evaluate the effects of different compounds on mean survival time in the heat-shock life span assay and bending frequency. Probability values of $P < 0.05$ were considered significant.

Results and discussion

mHtt protein aggregation in *C. elegans* inhibits development, motility, and survival

In the *C. elegans* model, we established that polyQ-GFP proteins tagged with GFP could be observed directly in

muscle cells under a fluorescence microscope. The animals expressing the normal polyQ repeat, Q16, showed smooth green lines in both the ventral and the dorsal muscle cells, while Q148-expressing nematodes showed discontinuous foci in muscle cells throughout their life span.³¹ The foci were mHtt aggregations, presenting as dots or even fibril-like strings (Figure 2A and B). In contrast to the relatively normal presentation of Q16-expressing worms, Q148-expressing worms were much shorter at the same stage of development (Figure 2A). They also had a shorter life span and weakened motility. The life span of Q148 worms decreased under heat stress, and total mortality was observed after heat treatment for 7 hours, whereas 30% of Q16 worms remained alive at that time (Figure 2C). Furthermore, Q148 worms also had a defect in bending, which can be reduced up to 70% in frequency in contrast to Q16 worms (Figure 2D).

These findings are in accordance with previously reported length-dependent toxicity of polyQ repeats. As lengthy polyQ tracts are more likely to cause binding between mHtt monomers, aggregates would accumulate, leading to the loss of original protein function and the gain of aggregated mHtt toxin;^{32,33} such behavior results in the deficits exhibited in the Q148 *C. elegans* model.

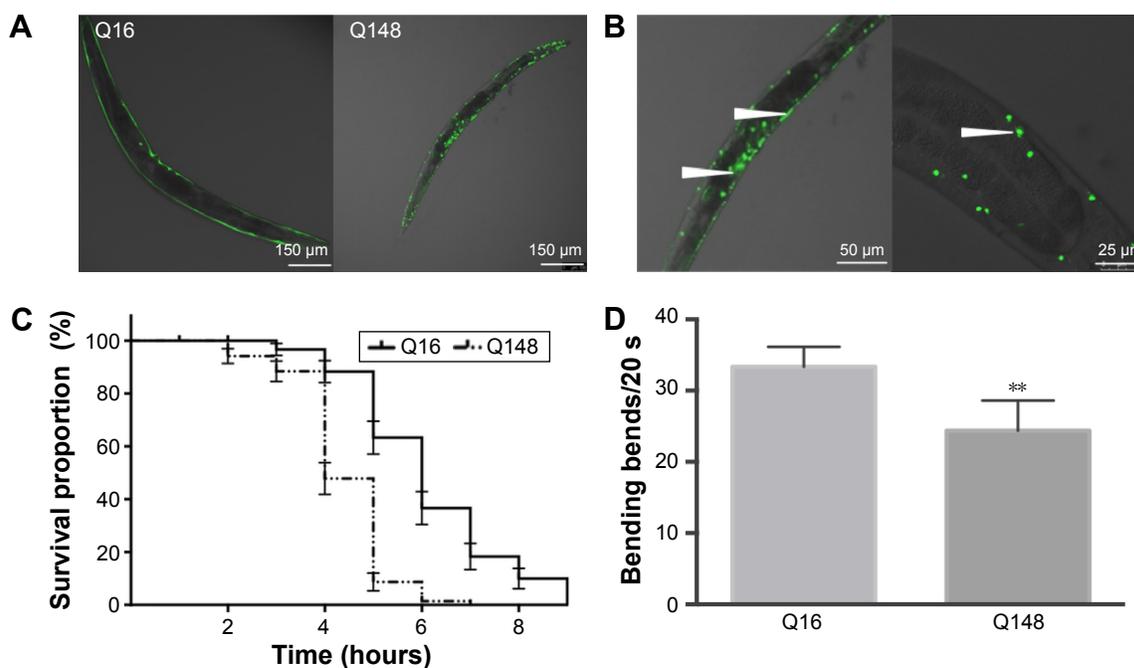


Figure 2 PolyQ-GFP protein distribution, survival rate and bending frequency in *C. elegans* Huntington disease model, carrying different length of polyQ (Q16 and Q148). **Notes:** (A) Aggregates can be seen all over the body wall muscle cells in Q148 worms. (B) The arrows indicated magnification of the aggregates existing as “dots” that gather to the form of “strings” in Q148 worms. (C) The average life spans were: Q16: 5.44 h, Q148: 4.95 h^{**}. (D) The average bending frequencies were: Q16: 32.33/20 s, Q148: 24.36/20 s. (** $P < 0.001$).

Abbreviations: PolyQ, polyglutamine; GFP, green fluorescent protein.

Xyloketal derivatives attenuate Q148-mediated defects in *C. elegans*

As expected, Q148 worms displayed a clear phenotype, providing a suitable model to examine the potential anti-mHtt toxin properties of xyloketals. All the Q148 model worms exposed to xyloketal compounds 1–6 showed marked improvement in the bioactivity assays; all six derivatives prolonged the life span under stress and improved motility (Figure 3A and B).

Notably, compound 1 reduced mortality in the first hour by 36% and improved motor function and growth development. Compared with the DMSO-treated worms (negative control), worms treated with compound 1 showed a 14% increase in the bending frequency. Also, no distinct shortening of body size was observed. Compounds 2 and 3 were found to have an extraordinary effect on the impaired motility of Q148; worms treated with these compounds showed nearly 38% increase in the bending frequency. Mean survival time was extended by nearly 3 hours on treatment with compound 3 relative to DMSO treatment. Compounds 4, 5, and 6 could effectively mitigate the PolyQ-mediated phenotype. Compound 5 showed the most significant effect among these three, with 50% and 35% improvements in heat-shock life span and bending assays, respectively, relative to the DMSO treatment. Overall, xyloketal derivatives 1–6 demonstrated a clear protective effect in the *C. elegans* model of HD, in both the heat-shock life span and bending assays.

Xyloketal derivatives 1, 4, 5, and 6 prevent mHtt aggregate formation

As previously reported, polyQ pathogenesis indicates that cellular dysfunction is often accompanied by aggregation

of mHtt.³⁴ Consistent with this, the formation of visible polyQ aggregate dots correlated with muscle dysfunction in transgenic worms bearing Q148. To explore whether xyloketals exert their protective effect by inhibiting mHtt aggregation, we examined the aggregate foci in Q148 worms after treatment.

The number of foci in body muscle cells was assessed in late-L4-stage worms. The protective effect of compounds 1, 4, 5, and 6 was accompanied by a reduction of aggregation in muscle cells. After 60 hours of treatment, the numbers of green foci located along the body wall cells decreased and fibril-like strings significantly reduced. The intensity of foci also weakened after treatment (Figure 4A). All these compounds diminished the aggregate foci to varying degrees, with 1 being the most effective. Statistical analysis revealed that compound 1 could decrease the aggregation by 20%, while compounds 4, 5, and 6 showed an average reduction of 10% (Figure 4B). However, compounds 2 and 3 showed no significant effect in the aggregation assay, which could suggest a different mechanism for their protective effects. Overexpression of mHtt has been known to change the chaperone activity and the global proteostasis network of *C. elegans*, and xyloketals might have protective properties mediated through these pathways.³⁵ Therefore, it is possible that compounds 2 and 3 protected the muscle cells by alerting the proteostasis network either singularly or in parallel with other molecules to clear aggregates. Western blotting showed that the formation of mHtt aggregates was affected by treatment with different compounds (Figure 4C).

For compounds 1, 4, 5, and 6, the decrease in mHtt aggregation in worm muscle cells was in accordance with the functional recovery these compounds promoted in

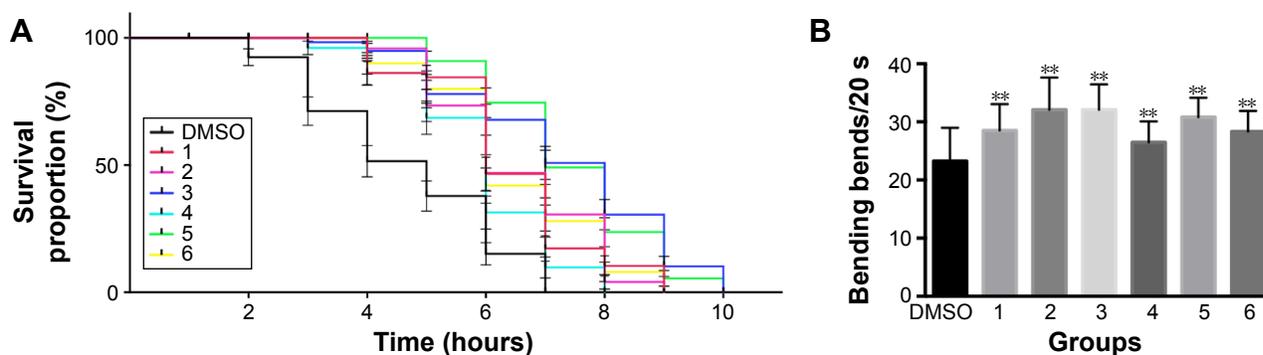


Figure 3 Effect of xyloketal derivatives on stressed life span and motility in the *C. elegans* Huntington's disease model.

Notes: (A) Survival analysis of worms treated by compounds (or DMSO as negative control) using the Kaplan-Meier method. The life span curve shows a time-dependent cumulative survival rate. The average survival time was: 1 (6.91 h**), 2 (6.86 h**), 3 (8.00 h**), 4 (6.50 h**), 5 (7.96 h**), 6 (6.79 h**), DMSO (5.05 h) respectively. (B) Bending frequency analysis was measured by the bending behavior over 20 s of worms in M9 buffer and was accomplished by ANOVA. The average bending counts were: 1 (28.5**), 2 (32.1**), 3 (32.1**), 4 (26.5**), 5 (31.4**), 6 (28.3**), DMSO (23.3). ** $P < 0.001$.

Abbreviations: *C. elegans*, *Caenorhabditis elegans*; HD, Huntington's disease; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.

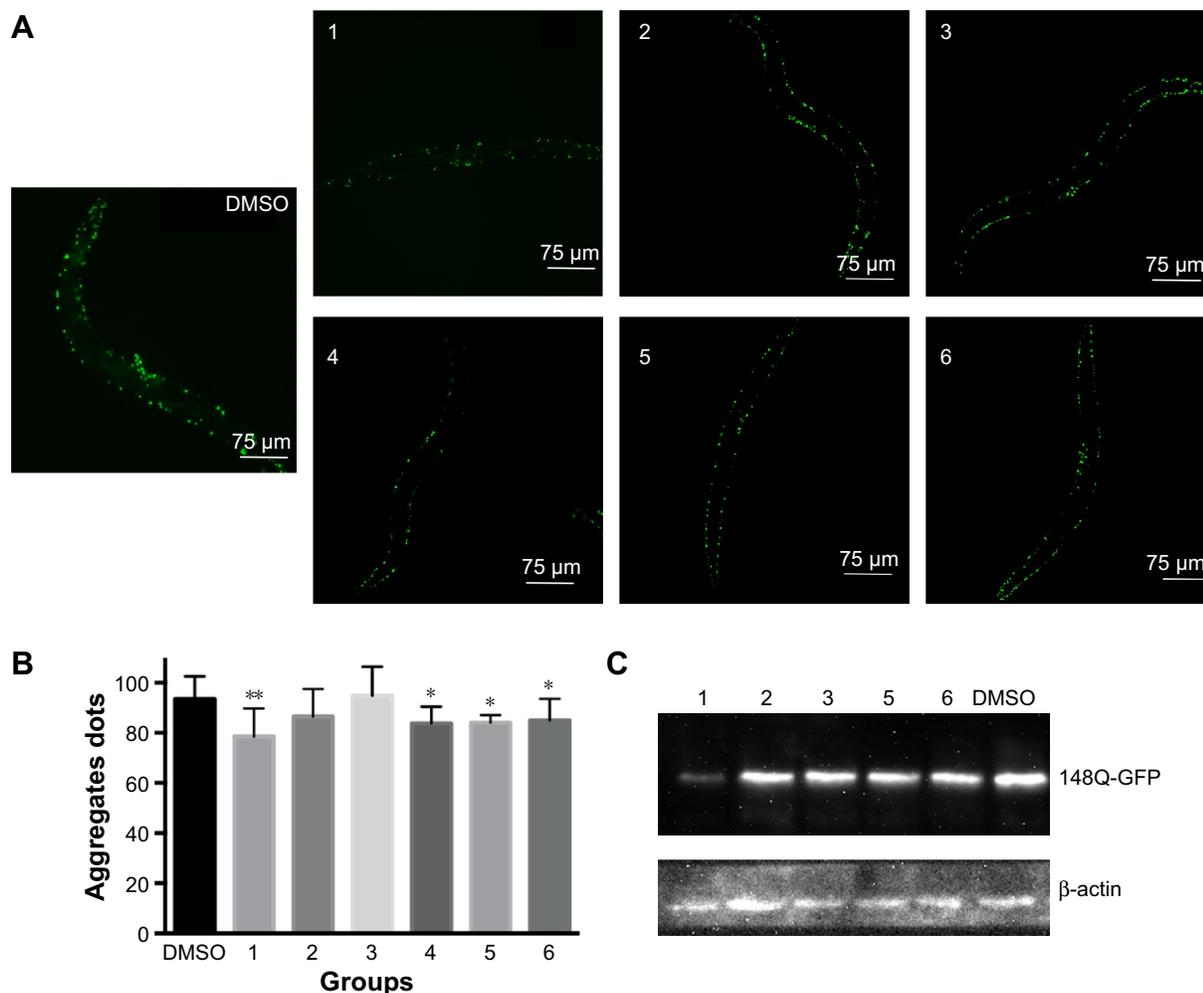


Figure 4 Effect of xyloketal derivatives on Htt aggregation.

Notes: (A) Worms treated with 100 μ M DMSO and other 100 μ M compounds (1–6); both the number and the intensity of green foci decreased in drug-treated groups. Fewer fibril-like strings could be observed in drug-treated groups. (B) Aggregate foci numbers on treatment with the various compounds were: 1 (78.6**), 2 (86.6), 3 (94.9), 4 (83.8*), 5 (84.1*), 6 (84.9*) vs DMSO (93.6) respectively. (C) The western blot of worms treated by DMSO and other drugs. * $P < 0.05$; ** $P < 0.001$.

Abbreviations: DMSO, dimethyl sulfoxide; Htt, huntingtin.

the bioactivity assays, indicating that disruption of mHtt aggregation may be the major neuroprotective mechanism for these xyloketals. Based on our results, we hypothesized that the screened compounds might act as inhibitors of Htt aggregation. This theory was tested in silico.

Xyloketal derivatives target the GLN site of Htt protein

HD is an autosomal-dominant neurodegenerative disorder caused by a polyQ expansion (>35Q) in the first exon (EX1) of Htt protein. mHtt protein is thought to adopt one or more toxic conformations that are involved in pathogenic interactions in cells. However, the structure of mHtt is not known. To investigate the mechanism of interaction of xyloketals with mHtt, we simulated contact between various compounds and Htt in silico. Since the polyQ region of mHtt is critical for mHtt toxicity, but no structure is available, Htt36Q3H-EX1

trimers in the crystal X1 (PDB code 4EF8) were used to conduct the molecular docking analyses as previously reported.³⁶ The structures of MBP and 3A linker were removed for clarity. Molecules were sketched according to the procedure described in the “Materials and methods” section. Automatic docking was employed. Other parameters were established by default in the software. In in silico analysis, the most potent compound, 1, was able to form hydrogen bonds with GLN396 using two benzopyran oxygens and a carboxyl oxygen (bond distances: 1.91, 2.05, and 2.58 Å, respectively). There was also a hydrogen bond between a hydroxyl group and the carboxyl oxygen from GLN393 (2.24 Å) (Figure 5A and B). As a result, mHtt protein and compound 1 may link together to form a stable trimeric complex (Figure 5C and D), which may attenuate further protein aggregation by preventing further interactions between β -strands. Pathologically, polyQ protein is thought to adopt one or more conformations that are

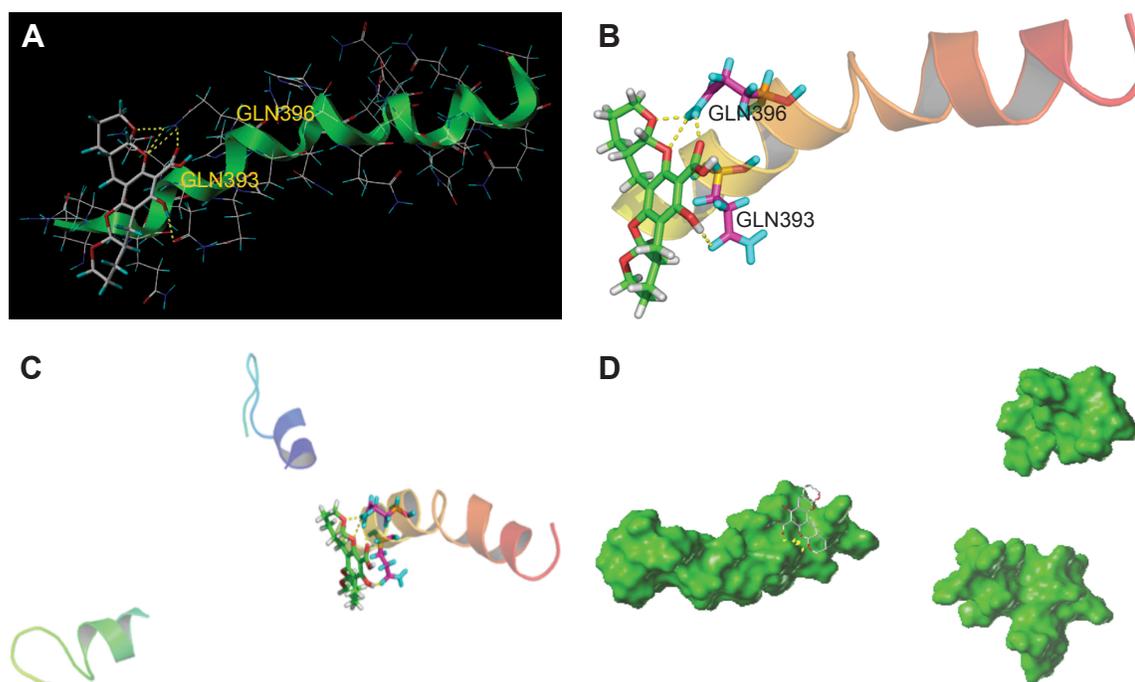


Figure 5 Potential target analysis in silico.

Notes: (A) and (B) The binding site of 36Q Htt and compound 1 is GLN396 and GLN393. (C) and (D) Interaction of Htt monomers can form stable trimer complex.

Abbreviation: Htt, Huntingtin.

involved in pathogenic interactions in cells.^{37–39} Compound 1 may abolish these conformations by interacting with GLN393 and GLN396 of the mHtt protein.

Docking scores for compounds 1–6 bound to mHtt showed varying capabilities in polyQ protein clearance (Table 1); greater docking scores indicate better clearance. The analysis results were in good agreement with functional recovery in the bioactivity assays described earlier, except for compound 6, probably because its smaller structure allows easy access into a cavity in the mHtt protein, leading to a distinct binding mechanism. Regardless of the specific mechanisms involved, our evidence supports the hypothesis that these compounds act as inhibitors of mHtt aggregation.

Consideration of the docking target analyzed earlier (GLN393 and GLN396) might suggest possible mechanisms for the antiaggregation properties of the xyloketal derivatives in the misfolding of mHtt. The glutamine residues of mHtt

can form polar zipper structures,^{40,41} which are part of β -strands, and can be linked together by hydrogen bonding between main-chain and side-chain amides.^{42,43} As the number of polyQ residues increases, these expanded sequences will show more affinity for each other and hold together in successive turns to form β -helix tubes.^{44,45} As the trimer complex of xyloketal-mHtt is formed, the secondary and tertiary structure of the mHtt protein may change. Formation of this complex could prevent self-propagation of mHtt aggregates via the polyQ sequences by influencing hydrogen bonding. As a result, the affinity for self-assembled formations of polyQ would decrease, which in turn might weaken the stability of the β -helix tubes. Consequently, the basic structure of mHtt aggregates will be interrupted by this process.⁴⁶

Conclusion

HD is a monogenic disease. The abnormal extension of CAG nucleotide sequences in the *IT15* gene seems to be the only key determinant. Disease severity and onset age is strongly associated with CAG triplet repeat number. Larger Htt protein, with abundant glutamine (Q), is more likely to form toxic aggregates. In the HD *C. elegans* model, all the tested xyloketal B derivative compounds were found to have a protective effect, which was mainly related to the attenuation of Htt aggregate formation. By simulating mHtt protein and compound interaction in silico, we demonstrated

Table 1 Ligand-Htt binding statistics

Ligand	Total score	Crash
1	5.44	-1.07
2	3.44	-0.97
3	3.01	-0.96
4	4.67	-1.10
5	4.35	-0.81
6	3.48	-0.31

Abbreviation: Htt, Huntingtin.

that the compounds could specifically bind to GLN residues in mHtt to prevent the proteins from forming bigger aggregates and therefore restrain further degeneration. Preventing aggregation of mutant proteins may be an attractive treatment strategy. As a novel drug candidate, xyloketal could be a promising therapeutic tool for both HD and other neurodegenerative diseases involving misfolded proteins.

In light of these findings, we may consider docked conformations of compounds with proteins to identify compounds with similar activities for future drug discovery. This strategy can significantly benefit the analysis of the potential therapeutic efficiency of drugs. By performing docking studies beforehand, we may guide new synthesis of compounds that target specific protein-binding sites. The same strategy can be applied to other neurodegenerative diseases with similar pathology of amyloidosis, such as Parkinson's, Alzheimer's, and prion diseases.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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