Inhibition of Huntingtin Exon-1 Aggregation by the Molecular Tweezer CLR01


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9rief Information

ABSTRACT: Huntington’s disease is a neurodegenerative disorder associated with the expansion of the polyglutamine tract in the exon-1 domain of the huntingtin protein (htt).1 Above a threshold of 37 glutamine residues, htt starts to aggregate in a nucleation-dependent manner. A 17-residue N-terminal fragment of htt (N17) has been suggested to play a crucial role in modulating the aggregation propensity and toxicity of htt.2 Here we identify N17 as a potential target for novel therapeutic intervention using the molecular tweezer CLR01. A combination of biochemical experiments and computer simulations shows that binding of CLR01 induces structural rearrangements within the htt monomer and inhibits htt aggregation, underpinning the key role of N17 in modulating htt toxicity.

Huntington’s disease (HD) is a neurodegenerative disorder characterized by the formation of misfolded proteins, which accumulate and eventually form amyloid aggregates in susceptible neurons. In HD, misfolding and aggregation is associated with the expansion of the polyglutamine (polyQ) tract in the huntingtin protein (htt).1 Htt proteins with polyQ expansions beyond 37 glutamine residues are known to be pathogenic.2 These polyQ repeats are located in the exon-1 domain of htt (htt1). Recent studies indicate that the regions flanking the polyQ segment significantly affect the aggregation process.3 These flanking regions are a 17-residue N-terminal domain of htt (N17) and a 52-residue region at the C-terminus with two polyproline (polyP) segments. The N17 sequence is a crucial part of the htt protein, as it is proposed to mediate a variety of targeting, trafficking, and clearing operations in the cell.4 Furthermore, it was shown that the N17 domain promotes the aggregation of htt in vitro and in vivo.5,6 In agreement with this, the specific binding of molecular chaperones to N17 was found to alter htt aggregation and toxicity.7

Here our aim was to modulate the aggregation propensity and cytotoxicity of htt using CLR01 (Figure 1A), a molecular tweezer that can inhibit the self-assembly and toxicity of several amyloid proteins.7 CLR01 binds specifically to lysine with a $K_d$ of $\sim 10 \mu M$ and to a lesser extent to arginine, while showing little or no affinity to other residues.8 This is important since lysine residues were previously implicated in htt aggregation.9 Also, CLR01 can be delivered to the central nervous system in mice.10 Notably, despite the well-known effect of CLR01 on other amyloid proteins, no studies of molecular tweezers on htt have been reported. This can be related to the small number of lysine residues found in htt, their concentration in the N17 sequence, and the fact that htt toxicity has been traditionally associated with the polyQ extension, where CLR01 is not expected to bind.

Here we show that CLR01 can indeed inhibit toxic aggregation of the htt protein. Our study reveals that CLR01 binds to the N17 domain of htt, thereby triggering structural rearrangements. Furthermore, we found that CLR01 induces a deceleration of the aggregation process in vitro and in living cells. To analyze the effect of CLR01 at the molecular level, we performed classical molecular dynamics (MD) and replica-exchange MD (REMD) simulations as well as quantum mechanics/molecular mechanics (QM/MM) calculations of htt with 55 glutamine residues (htt55Q55). The structure reported by Dlugosz and Trylska12 was used as a starting point for MD simulations of httQ55 in the absence of tweezers. A cluster analysis of this trajectory was...
performed to identify the most populated structures. These structures, (CS1, CS2, or CS3; Figure S1) were used for the simulations with CLR01 or with the negative control molecule CLR03 (Figure 1A). Of the three lysine residues found in N17 of httQ55 (Lys6, Lys9, and Lys15), the QM energies from the QM/MM calculations (DFT-D3/CHARMM) suggest that the tweezer–lysine interaction is most favorable when CLR01 binds to Lys9 (Table S1 and Figure S2). However, as evidenced by the MD simulations, N17 can accommodate three CLR01 molecules simultaneously. Next, we investigated the effect of CLR01 on httQ55, MD simulations of CS1, CS2, and CS3 at 300 K in explicit solvent did not report changes in the secondary structure content of the polylQ55 region in the presence of CLR01 (Figure S3). The opposite was true for the N17 domain. Without CLR01, N17 has high α-helical content (∼81% in CS1; Figure 1B, left). The three most populated averaged structures (∼71% of the total population) of the N17 domain of CS1 show a two-helix bundle fold (SI, Figure S4A). These results are in line with previous reports on the tendency of the N17 domain to adopt such a conformation.12,13

The results for CS1 without tweezer were also corroborated by simulations of CS2 and CS3 (SI, Figure S5). Next, we analyzed the influence of CLR01 on the N17 region. We found that binding of CLR01 to the lysine residues induced significant changes in the secondary structure content of N17. The α-helical content in residues 1–9 decreases on average by ∼25% in CS1 (Figure 1B, right). This effect was also observed for CS2 and CS3, although to a lower extent (SI, Figure S5). The averaged structures of the N17 domain of CS1 interacting with CLR01 evidenced the loss of α-helical content (SI, Figure S4B). Simulations with CLR03 did not show such influence on the secondary structure propensity of N17 (SI, Figures S6 and S7).

Changes in the α-helical content of N17 were also observed in 1:1 simulations, where only one CLR01 molecule interacted with one of the three lysine residues (Figure S8). Although the effect was less pronounced than in the 1:3 httQ55–CLR01 complexes, in all of the 1:1 cases the decrease in helicity was larger than when three CLR03 molecules were simultaneously used. To further assess our results, REMD simulations of CS1 were carried out in explicit solvent with a different force field (see the Supporting Information (SI)). Under these different simulation conditions, a substantial decrease in the α-helical content of the N17 domain was also observed upon binding of three CLR01 molecules to httQ55 (Figure 2).

We studied the CLR01–N17 binding event by native electrospray ionization mass spectrometry (ESI-MS) and detected a 1:1 N17–CLR01 complex with an estimated Kd of 20 μM (Figure 1C) and no binding of CLR03. Further, tandem mass spectrometry (MS/MS) of the N17–CLR01 complex using electron capture dissociation (ECD) allowed us to determine the binding sites. ECD-MS/MS of peptide–ligand complexes yields fragment ions of the peptide chain, some of which are still bound to the ligand. The sequence of these ligand-bound fragments was used to analyze the ligand-binding regions.14 In agreement with the simulations, Lys9 was determined to be the most probable binding site (SI, Figure S9). Isothermal titration calorimetry (ITC) experiments produced biphasic curves that revealed complexation of at least two binding sites (Figure 1D) with titration data resembling those from previous studies of CLR01 binding to a protein with multiple sites.15 No heat change was observed in CLR03 experiments (SI, Figure S10). We further used circular dichroism (CD) spectroscopy to study changes in the secondary structure. CLR01 was added to httQ55 at a protein:CLR01 ratio of 1:10 or

![Figure 1. (A) Chemical structures (side view) and computed geometries (front view) of the tweezer CLR01 and the spacer CLR03. (B) Secondary structure content of the calculated structures of the N17 domain of the httQ55 conformer CS1 in the absence (left) or presence (right) of CLR01. (C) ESI mass spectrum of the N17 peptide and CLR01. (D) ITC experiment of the N17 peptide with CLR01 injections. Inset: Baseline corrected raw data. (E) Normalized CD spectra of httQ55 with CLR01 or CLR03 (to 207 nm for comparison).11 Inset: Helical content. One-way ANOVA and Bonferroni posthoc tests were performed in comparison with the buffer control: ns, P > 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.](https://doi.org/10.1021/jacs.6b11039)
Upon addition of CLR01, a decrease in the \( \alpha \)-helical content was observed in a concentration-dependent manner, while the shape of the CD spectrum remained unchanged upon addition of CLR03. These observations agree with the modeling results.

The N17 domain has been described previously as amphipathic. As CLR01 induced a major conformational change in this domain, we asked whether CLR01 also affects the amphipathic nature of N17. In the absence of CLR01, N17 formed an amphipathic structure with a patch of hydrophobic residues opposite to a patch of charged residues (Figure 3A). Such hydrophobic patches are known to interact with similar hydrophobic cores of the N17 domain of other monomers, thereby enhancing aggregation. Our analysis revealed that CLR01 binding disrupts the hydrophobic patch (Figure 3B). In contrast, with CLR03 the amphipathic character of N17 was less affected (Figure 3C). A similar tendency was found in the REMD simulations (SI, Figure S12).

As discussed above, our in silico experiments indicated that binding of CLR01 disrupts the helical structure and the amphipathic nature of the N17 domain. Consequently, CLR01 binding would be expected to influence the ability of the N17 domain to self-associate, thereby modulating the aggregation of the entire htt\(^{Q55}\). To test this hypothesis, we performed a thioflavin T (ThT) assay in the presence and absence of CLR01.

The lag time of aggregation \( (T_{\text{Lag}}) \) was obtained by fitting a nucleated growth model to kinetic traces. Addition of CLR01 caused a dose-dependent decrease in the amount of fibrils formed (Figure 4A) as well as an increase in \( T_{\text{Lag}} \) (Figure 4B).

Addition of CLR03 slightly decreased \( T_{\text{Lag}} \) in agreement with earlier studies showing that small molecules such as ThT and CLR03 may template aggregation or facilitate oligomerization. This effect could also explain the minor offset of the CD spectra observed for CLR03 addition to htt\(^{Q55}\) (SI, Figure S11). Furthermore, we used a recently developed in-cell kinetic aggregation assay in which htt\(^{Q72}\) PC12 cells treated with CLR01/03 for 48 h after transfection (\( n = 16–19 \) and \( n = 9 \), respectively). Two-way ANOVA and Tukey posthoc tests were performed pairwise (labeled with \(*\) and to the reference without compounds (dotted line, labeled with \(#\)).

Figure 2. Replica Exchange Molecular Dynamics (REMD) simulations also show a decrease in the \( \alpha \)-helical content of the N17 domain upon binding of three CLR01 molecules. Representative structures (most populated clusters) and secondary structure contents of N17 A) without tweezer, B) with CLR01.

Figure 3. Distribution of hydrophobic (blue) and hydrophilic (red) residues on the surface of the N17 domain (A) without compound, (B) with CLR01, and (C) with CLR03.

Figure 4. Effect of CLR01/03 on htt\(^{Q14}\) aggregation, cellular morphology, and viability. (A) ThT assay. Each trace represents mean ± standard deviation, \( n = 3 \). (B) Kinetic lag times. (C) In-cell aggregation kinetics (representative single-cell curves). (D) Statistical analysis of the kinetic lag times in different HeLa cells (htt\(^{Q52}\), \( n = 32 \); htt\(^{Q72}\) CLR01, \( n = 31 \); htt\(^{Q72}\) CLR03, \( n = 12 \)). One-way ANOVA and Bonferroni posthoc tests were performed in comparison with the untreated control. (E, F) Neurite length and cell viability of htt\(^{Q103}\) PC12 cells treated with CLR01/03 for 48 h after transfection (\( n = 16–19 \) and \( n = 9 \), respectively). Two-way ANOVA and Tukey posthoc tests were performed pairwise (labeled with \(*\) and to the reference without compounds (dotted line, labeled with \(#\)).
decreased $T_{1/2}$ by 5 min, in agreement with the ThT experiments (Figure 4C,D). Treatment of the HeLa cells with either compound at up to 100 μM had no effect on the cell viability (Figure S13). We further tested the effects of CLR01 and CLR03 using neuronal PC12 cells expressing httQ103 (Figure S14). Outprisingly, we found a protective effect on neurite length of cells treated with 1 μM and 10 μM CLR01 for 48 h after induction (Figure 4E). However, treatment with 100 μM CLR01 caused a decrease in cell count (Figure S15A), indicating an enrichment of toxic soluble species inside the cells.23 Remarkably, an increase in cell viability was observed with 100 μM CLR03 (Figure 4F), which can be explained by an increase in proliferation (Figure S15B). The mechanism of this beneficial effect is yet unknown. Treatment with CLR01 or CLR03 decreased the number of aggregate-containing cells and increased the aggregate sizes (Figure S15C,D). In agreement with the kinetic in-cell assay, this suggests that CLR01 does not prevent inclusion body formation per se but rather decelerates the aggregation kinetics and inhibits fibril formation (Figure 4A–D).

In summary, our studies have revealed that binding of CLR01 to the lysine residues of N17 leads to a loss of α-helical content in the N17 domain, inhibiting fibril formation and decelerating aggregation with beneficial effects on neurite growth. Our work thus presents a novel therapeutic approach for treatment of HD.

References


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