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Inhibiting nucleation of amyloid structure in a huntingtin fragment by targeting α -helix rich oligomeric intermediates

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Abstract

Although oligomeric intermediates are transiently formed in almost all known amyloid assembly reactions, their mechanistic roles are poorly understood. Recently we demonstrated a critical role for the 17 amino acid N-terminal segment (htt^{NT}) of huntingtin (htt) in oligomer-mediated amyloid assembly of htt N-terminal fragments. In this mechanism, the htt^{NT} segment forms the α -helix rich core of the oligomers, leaving most or all of each polyglutamine (polyQ) segment disordered and solvent-exposed. Nucleation of amyloid structure occurs within this local high concentration of disordered polyQ. Here we demonstrate the kinetic importance of htt^{NT} self-assembly by describing inhibitory htt^{NT}-containing peptides that appear to work by targeting nucleation within the oligomer fraction. These molecules inhibit amyloid nucleation by forming mixed oligomers with the htt^{NT} domains of polyQ-containing htt N-terminal fragments. In one class of inhibitor, nucleation is passively suppressed due to the reduced local concentration of polyQ within the mixed oligomer. In the other class, nucleation is actively suppressed by a proline-rich polyQ segment covalently attached to htt^{NT}. Studies with D-amino acid and scrambled sequence versions of htt^{NT} suggest that inhibition activity is strongly linked to the propensity of inhibitory peptides to make amphipathic α -helices. Htt^{NT} derivatives with C-terminal cell penetrating peptide segments, also exhibit excellent inhibitory activity. The htt^{NT}-based peptides described here, especially those with protease-resistant D-amino acids and/or with cell penetrating sequences, may prove useful as lead therapeutics for inhibiting nucleation of amyloid formation in Huntington's disease.

Keywords

amyloid; polyglutamine; inhibitor; α -helical intermediate; cell penetrating peptide

In Huntington's disease (HD), inheritance of an expanded version of a polyglutamine (polyQ) encoding CAG repeat in the gene for the ~ 3,200 amino acid huntingtin (htt) protein is tightly linked to devastating neurodegeneration¹. As in the case of the eight or nine² other expanded CAG repeat diseases, various aggregated forms (i.e., larger than monomer)

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of htt are found in brain tissue from patients and from experimental animals^{3; 4}. A strong correlation in vitro^{5; 6} and in vivo⁷ between polyQ repeat expansion and aggregation provided further links between pathology and protein misfolding and/or aggregation^{8; 9; 10}. Many studies in a variety of mutant htt-expressing model organisms, including transgenic mice^{11; 12; 13; 14; 15}, report the detection in the brain of insoluble and/or aggregated forms of huntingtin occurring roughly in the same time frames as the development of disease symptoms. Although correlations between the appearance of aggregates and of pathology in cell and animal models are not universally observed¹⁶, this may be due in part to current limitations on our ability to observe micro-aggregates in cells and/or tissues. In any case, it is clear that more work must be done to further probe the roles of aggregated huntingtin in the HD mechanism. One approach to testing this hypothesis is to develop tools for preventing or changing the course of aggregation in cell and animal models.

The N-terminal 3% of the huntingtin protein consists of a 17-amino acid N-terminus (the htt^{NT} segment) followed by the polyQ repeat, followed by a proline-rich segment¹¹. The remaining 97% of htt appears to be rich in HEAT repeats often involved in assembly of protein complexes, but has no other evident folding units^{17; 18}. Proteolytic events that release, from full-length htt, N-terminal fragments that contain the htt^{NT}, polyQ and Pro-rich segments have been implicated as an early step in pathogenesis^{19; 20}, and transgenic animals expressing similar short, N-terminal fragments exhibit particularly aggressive neuropathology^{1; 21}. These observations together suggest that some property of htt that is enhanced in polyQ-containing N-terminal fragments must play an important role in disease.

Simple polyQ peptides aggregate according to a classical nucleated growth polymerization mechanism with no non-amyloid intermediates^{22; 23; 24; 25; 26; 27}. Some polyQ flanking sequences, however, can substantially alter polyQ aggregation mechanism and/or kinetics^{28; 29; 30; 31; 32; 33}. In particular, the N-terminal htt^{NT} segment of htt has a dramatic enhancing effect on the aggregation and toxicity of polyQ.

The htt^{NT} segment possesses a mix of hydrophobic and hydrophilic amino acids (Table 1) and is predicted to have both significant α -helix potential^{34; 35; 36} and a moderate tendency toward compact structure³², two characteristics typical of MoRF sequences³⁷. MoRF (Molecular Recognition Features) sequence elements exist in isolation as intrinsically disordered elements, but can acquire structure in the presence of a binding partner³⁸. Thus, proton NMR shows that there is no stable secondary structure in this peptide in native aqueous solutions³², but CD spectra^{32; 34} and MD simulations³⁶ suggest that the htt^{NT} sequence by itself has a tendency to take on some α -helical secondary structure. In spite of, or perhaps because of, this ambiguous structure, the short htt^{NT} sequence appears to play an important role in the biological life of the huntingtin protein. Post-translational modifications of the htt^{NT} sequence in full-length or N-terminal fragments of htt, in cell and animal models, have been implicated in playing important roles in normal and abnormal htt cell biology and in htt pathology^{15; 35; 39; 40; 41}.

Consistent with this, the htt^{NT} segment exhibits a striking effect on polyQ aggregation^{32; 42}. Peptides with the structure htt^{NT}Q_N aggregate with much higher rates than the identical repeat length simple polyQ sequences at similar concentrations³². They accomplish this *via* a radically altered aggregation mechanism. Thus, htt^{NT}Q_N peptides first assemble into small, oligomeric structures that serve as the medium within which nucleation of amyloid structure occurs³². In these oligomers, consistent with their MoRF-like character, the htt^{NT} segments self-associate to form an α -helix rich⁴³(Jayaraman et al., Ms. submitted) core from which unstructured polyQ is largely excluded³². Presumably facilitated by the high local concentration of disordered polyQ, amyloid structure is nucleated within some of these

oligomers, leading to a burst in aggregation rate³² and a polyQ-repeat length dependent transition from α -helix-rich to β -sheet-rich aggregate (M. Jayaraman et al., Ms. submitted).

In this paper we show that the aggregation of htt N-terminal fragments can be strongly inhibited by molecules that compromise the role these α -helix rich oligomers play in the aggregation mechanism. We show that the htt^{NT} peptide itself, with or without very short polyQ extensions, co-assembles with htt^{NT}Q_N peptides to make mixed oligomers whose nucleation efficiency is much reduced, presumably due to the reduction in the local concentration of polyQ within the oligomers. We also show that even greater inhibition can be obtained if a Pro-containing polyQ sequence²³ is attached to the htt^{NT}, creating a molecule that can co-assemble into oligomers with htt N-terminal fragments where it actively inhibits polyQ amyloid nucleation. Further experiments with sequence analogs of htt^{NT} suggest that htt^{NT} co-assembly depends on the ability of this sequence to form amphipathic α -helix. These htt^{NT}-related molecules exhibit a novel means of inhibiting htt amyloid assembly, providing insights into the normal aggregation mechanism as well as potential tools for both understanding the molecular basis of expanded polyQ pathogenicity and for drug discovery. In fact, modifications designed to improve the cell uptake and stability of these peptides were found to not disrupt their inhibitory activities.

RESULTS

After discovering that the htt^{NT} component in *cis* confers onto polyQ a dramatic increase in aggregation kinetics with a substantial change in mechanism³², we conducted experiments to test whether this effect might operate in *trans* as well. An example of the strong *cis* effect is shown in Figure 1a, where the htt N-terminal fragment htt^{NT}Q₃₇P₁₀K₂ (●) aggregates to completion over a period of a few hours, while similar peptides lacking some (▲) or all (▲) of the htt^{NT} sequence do not progress beyond 20% aggregation even after 120 hrs. In contrast, we observe no *trans* effect, in that an equimolar amount of htt^{NT} incubated with the K₂Q₄₁K₂ peptide (■) produces no change in the relatively slow aggregation rate seen for the K₂Q₄₁K₂ peptide alone (♦) (Fig. 1a). This latter result is in contrast to a recent report claiming such a *trans* effect⁴². Because this recent contradictory report was based on a significantly modified polyQ peptide⁴², we considered the possibility that the discrepancy in results might derive from major differences in the sequence context of the polyQ peptides in the two studies. We therefore obtained by chemical synthesis exactly the same sequence reported to be enhanced by htt^{NT} in *trans*⁴² and studied its aggregation. We found that the peptide alone aggregates slowly (Fig. 1b, ●), at an initial rate that is comparable to that of a simple polyQ peptide of similar repeat length and concentration²⁷. We also found, however, that equimolar htt^{NT}, if anything, slightly inhibits aggregation of this peptide (Fig. 1b, ●), rather than enhancing it. It is not clear why the results of Tam et al.⁴² differ from ours; it might be due to differences in peptide handling or substantial differences in the aggregation assay.

In any case, as a control in the Figure 1 experiments, we also incubated htt^{NT} with the htt N-terminal fragment htt^{NT}Q₃₀P₆K₂ and found, surprisingly, that htt^{NT} inhibits aggregation of this htt N-terminal fragment in a concentration-dependent manner (Fig. 2a). Furthermore, early in the inhibited reaction, a portion of the htt^{NT} inhibitor becomes susceptible to sedimentation (Fig. 2b), suggesting possible formation of a co-aggregate between htt^{NT} and htt^{NT}Q₃₀P₆K₂ (compare Fig. 2b to Fig. 2a). After this initial drop, little or no additional htt^{NT} co-aggregates. Note that in the absence of htt^{NT}Q₃₀P₆K₂, very little htt^{NT} aggregates under these conditions (Fig. 2b, ●). The inhibition of htt^{NT}Q₃₀P₆K₂ aggregation by htt^{NT} can be characterized as a delay in the inevitable; that is, although increasing concentrations of htt^{NT} lead to longer delay times in the aggregation reaction, robust aggregation nonetheless eventually occurs, going essentially to completion regardless of the amount of

htt^{NT} inhibitor present (Fig. 2a). This is also true when a stoichiometric excess of htt^{NT} over htt^{NT}Q₃₀P₆K₂ is used (Fig. 2c). Further, multiple equimolar doses of htt^{NT} given over the reaction time course further slow aggregation, so that, within the reaction time monitored, amyloid formation did not go to completion (Fig. 2c, ■).

Mechanism of inhibition

To probe the mechanism of these inhibitory effects, we first tested whether htt^{NT} can form mixed low MW co-oligomers, such as heterodimer, with htt^{NT}Q₃₀P₆K₂. We incubated a mixture of these two peptides and subjected the mixture to size exclusion chromatography (SEC) with the column fractions being subjected to LC—MS analysis (Fig. 3). The SEC profile shows two major peaks eluting at the MW positions of monomeric htt^{NT}Q₃₀P₆K₂ and htt^{NT} (Fig. 3a). Each peak was collected and injected into an analytical reverse phase HPLC column with MS detection. We found that the early eluting SEC peak (Fig. 3a) gives LC-MS data consistent with homogeneous htt^{NT}Q₃₀P₆K₂ (Fig. 3b), and the later eluting peak gives data consistent with homogeneous htt^{NT} (Fig. 3c). Thus, there is no evidence for stable low MW complexes of these two proteins, which, if present, should elute earlier than htt^{NT}Q₃₀P₆K₂ in Figure 3a and give LC-MS signals for both peptides in the same SEC fraction. This experiment does not rule out the possible existence of stable complexes that do not chromatograph well, or of less stable complexes that might disintegrate early in the SEC run time.

Although the above experiment suggests that these two peptides do not form stable, low MW complexes with each other, electron microscopy suggests that upon incubation they quickly form large spherical co-oligomers. As reported previously for other htt N-terminal fragments, we found that htt^{NT}Q₃₀P₆K₂ incubated alone forms small spherical oligomers within 15 mins of incubation (Fig. 4b), to be replaced at 5.5 hrs with small fibril structures (Fig. 4c) that eventually grow to intermediate fibrils at 24 hrs (Fig. 4d) and longer and thicker fibrils (Fig. 4e, f) at later times. In contrast, when co-incubated with htt^{NT}, some of the early oligomers are much larger (Fig. 4g, h) and persist long beyond 15 mins, exhibiting a mixture of oligomers (Fig. 4i, l) and fibrils (Fig. 4j, k) at both 5.5 (Fig. 4i, j) and 24 (Fig. 4k, l) hrs, before reaching a point of uniform fibril morphology at 48 (Fig. 4m) and 72 (Fig. 4n) hrs that is indistinguishable from htt^{NT}Q₃₀P₆K₂ alone (Fig. 4e, f). In contrast, as previously reported, htt^{NT} incubated by itself aggregates very slowly, forming oligomeric clusters only after hundreds of hours of incubation³². The uncharacteristically large oligomers uniquely formed rapidly in the htt^{NT} inhibited htt^{NT}Q₃₀P₆K₂ aggregation reaction suggest that these oligomers represent co-aggregates of the two component peptides.

Confirmatory evidence for co-aggregate formation comes from experiments with peptides containing an F17W replacement within the htt^{NT} component. Previously we used tryptophan intrinsic fluorescence to probe the burial of a Trp probe in aggregate structure³². Here, when we incubated a 1:1 mix of htt^{NT}Q₃₀P₆K₂ and htt^{NT} (F17W) for 4 hrs, a time frame when only oligomers are present by EM (Fig. 4g, h), we found that the isolated aggregates exhibit strong Trp fluorescence (Fig. 5a, blue) consistent with the presence of the htt^{NT} inhibitor in the aggregate. Since htt^{NT} peptides incubated in isolation aggregate only very slowly³², to find htt^{NT} (F17W) fluorescence in the early aggregate pool suggests that its aggregation has been somehow stimulated by the presence of htt^{NT}Q₃₀P₆K₂, and the simplest rationale for such stimulation is co-aggregation with htt^{NT}Q₃₀P₆K₂. This indirect evidence for an intimate co-aggregate is confirmed by the details of the fluorescence spectrum.

While Trp is solvent exposed (i.e., the fluorescence maximum is 355 nm, unshifted from that of the monomer³²) in oligomers slowly produced when htt^{NT} (F17W) is incubated alone³², the Trp fluorescence of rapidly formed aggregates isolated from the co-mixture

with htt^{NT}Q₃₀P₆K₂ (Fig. 5a, blue) is dramatically shifted to 337 nm, indicating a solvent-excluded environment for the Trp in these aggregates. These aggregates are almost certainly oligomeric (i.e., not fibrillar), since the time course of the fluorescence shift within the aggregate fraction goes essentially to completion within 5 hrs (Fig. 5b); in equivalent reactions, the aggregates at 5 hrs are mostly oligomeric (Fig. 4), and the rapid amyloid formation phase has not yet commenced (Fig. 2a). These data suggest that htt^{NT} (F17W) forms aggregates in this mixed reaction via an intimate oligomeric co-aggregate with htt^{NT}Q₃₀P₆K₂. Interestingly, the fluorescence maximum for the co-mixture of htt^{NT} and htt^{NT}Q₃₀P₆K₂ is much more shifted when Trp17 is in the inhibitor (Fig. 5a, blue, 337 nm) than when it is in the htt^{NT}Q₃₀P₆K₂ (Fig. 5a, magenta, 349 nm). Thus, the local environment around position 17 in the htt^{NT} inhibitor is solvent-excluded in co-aggregates with a huntingtin fragment, a qualitatively different result from either aggregates of htt^{NT} alone³² or co-aggregates in which it is the htt^{NT}Q₃₀P₆K₂ peptide that contains a Trp (Fig. 5a, magenta).

In contrast to these oligomeric intermediates, the final aggregated product of htt^{NT}-inhibited reactions is amyloid in nature, and is in fact indistinguishable from the product of the uninhibited reaction, by FTIR (Fig. 5c) as well as EM (Fig. 4). While, as previously reported⁴³, the slowly formed aggregates of htt^{NT} incubated alone are rich in α -helix by FTIR (Fig. 5c, black), the spectrum of htt^{NT}Q₃₀P₆K₂ aggregates (Fig. 5c, red) is dominated by the three bands characteristic of polyQ amyloid absorbing at 1604.6 cm⁻¹ (Gln side chain N-H bending), 1626 cm⁻¹ (β -sheet), and 1656.8 cm⁻¹ (Gln side chain C=O stretching). The FTIR spectrum of the final aggregated product of the htt^{NT}-inhibited reaction of htt^{NT}Q₃₀P₆K₂ (Fig. 5c, blue) is identical to that of the uninhibited aggregation product. (Because of the very small mass of oligomeric co-aggregates formed in the inhibited reactions, it has not been possible to obtain FTIR spectra on these pre-amyloid assemblies.)

These data support a model in which htt^{NT} inhibitors co-assemble with the htt^{NT} segments of htt^{NT}Q₃₀P₆K₂ peptides into mixed, α -helix rich oligomers, thereby decreasing the local concentration of exposed polyQ segments in comparison to homogeneous htt^{NT}Q₃₀P₆K₂ oligomers; this reduced local polyQ concentration restricts the polyQ interchain interactions that drive nucleation of amyloid structure³². To further characterize this proposed mechanism, we conducted a detailed series of experiments with structurally modified htt^{NT} analogs.

Structure-function relationships in htt^{NT} inhibition

To investigate the structural specificity of htt^{NT} inhibition, we obtained an all-D version of htt^{NT} as well as a “retro-inverso”⁴⁴ version of htt^{NT}, in which the htt^{NT} sequence is not only constructed with D amino acids but is also inverted (Table 1). Such retro-inverso peptides are sometimes used in an attempt to partially restore the side chain orientations of the wild type all-L peptide in an all-D peptide analog⁴⁴. {It should be noted that optimal retro-inverso sequences also include modifications at the N- and C- termini to restore the terminal charge identity, with respect to sequence, found in the parent, L-version⁴⁵; this was not done in our peptide.} Our expectation in designing this experiment was that there must be important stereochemical specificity in htt^{NT} mixed oligomer formation that would be compromised when D-htt^{NT} interacts with L-htt^{NT}Q_N. We were therefore surprised to find that both the all-D and retro-inverso versions of htt^{NT} are also effective inhibitors of L-htt N-terminal fragment aggregation (Fig. 6a). In this experiment we used the slower aggregating P₁₀ version of the Q₃₀ N-terminal fragment, which probably explains the apparently improved inhibition by the all-L htt^{NT} peptide in this experiment. In any case, under the same conditions, both the all-D and the retro-inverso versions of htt^{NT} are equally effective inhibitors of htt^{NT}Q₃₀P₁₀K₂ aggregation (Fig. 6a). Consistent with our hypothesis that inhibition requires the ability to form an α -helix rich oligomer, we found that the all-D

and retro-inverso forms of htt^{NT} form aggregates at the same slow rates (not shown) as seen previously for all-L htt^{NT} (Fig. 2b), and that FTIR spectra of the aggregates formed are identical to aggregates of all-L htt^{NT} (Fig. 5d) in suggesting highly α -helical structures.

One interpretation of this result is that the inhibition we observe with htt^{NT} has no real structural specificity, and may simply depend on the net hydrophobicity and charge characteristics of this collection of amino acids in a short peptide. To test this hypothesis, we designed and obtained a series of scrambled sequence variants of the inhibitory peptide htt^{NT}Q. A list of 49 random sequences (Supporting Table 1) was generated using MATLAB (Methods). This list was then visually edited (Methods) to produce 20 scrambled sequences with a variety of distributions of charged and hydrophobic residues (Supporting Table 1). These 20 sequences (Table 2) were obtained by custom synthesis and purified by HPLC (Methods). Some of the peptides gave inadequate yields after purification and were not pursued. In addition, two of the peptides co-migrated in HPLC with the htt N-terminal fragment aggregation substrate, complicating aggregation kinetics analysis, and were therefore not pursued. Three others readily grew into amyloid fibrils under PBS, 37 °C conditions and were not pursued as part of these experiments. After attrition of candidate peptides for the above reasons, we were left with 10 scrambled peptides (Table 2) amenable to further analysis, and these were tested, along with control htt^{NT}, as potential inhibitors of htt^{NT}Q₃₀P₆K₂ (Fig. 6b,c) at a 1:1 stoichiometric ratio. Of these 11 peptides, the wild type htt^{NT} sequence (○) proved to be the best inhibitor. Furthermore, five of the random peptides (peptides # 2, 4, 5, 6, and 9) exhibited no inhibitory activity under these conditions. Interestingly, however, the other five peptides (# 3, 7, 8, 11, and 12) gave good to very good inhibitory activity, with one inhibitor, peptide # 11, delaying the time to 50% aggregation almost as long as htt^{NT} itself (Fig. 6c; Table 2).

This scrambled sequence experiment produced the counter-intuitive result that while htt^{NT} inhibition is impervious to a universal L to D switch at the amino acid level (Fig. 6a), it nonetheless exhibits significant sequence selectivity within a series of scrambled sequences. One possible way to rationalize these two sets of results is the hypothesis that inhibition depends on the formation of an α -helix displaying a particularly effective pattern of surface charge and/or hydrophobicity, a condition unlikely to be equally achievable by all of scrambled sequences. To test this α -helix hypothesis, we first calculated an α -helix potential for each scrambled sequence using AGADIR⁴⁶ (Table 2). A plot of these values against the inhibitory activities (time to 50% aggregation; Table 2) of the each peptide (Fig. 7a) shows a good linear correlation with $R^2 = 0.80$. We next considered whether the amphipathicity or hydrophobic moments⁴⁷ of such α -helices might play a role in inhibitory activity. We calculated the hydrophobic moments (μ H) of α -helices populated by the different scrambled sequences (Table 2) and plotted the results against inhibitory activity. The results (Fig. 7b) show a very good correlation with $R^2 = 0.88$. Based on these encouraging results, we determined CD spectra of each scrambled peptide alone at 100 μ M and calculated the % α -helix (Methods) (Table 2). The actual predicted α -helix formation for each peptide was then used to normalize the hydrophobic moment values (which of course are obtained by assuming 100% α -helix formation) and the resulting “normalized μ H” values were plotted against inhibitory activity. The results (Fig. 7c) exhibit a good correlation with $R^2 = 0.70$. This latter plot features a very good correlation among peptides with very low α -helix potential, consistent with the importance of α -helix formation in the mechanism of inhibition. It also links the highest normalized μ H with the highest inhibitory activity, corresponding to the wild type sequence (●). At the same time, the higher departure from the fitted line among the more strongly inhibiting peptides shows that the calculated normalized μ H values have a lower predictive value in this regime. There are several possible explanations for this. It might be due to experimental uncertainty in the α -helix contents calculated from the CD spectra. It is also quite likely that estimates of α -helix

formation in these peptides as monomers in solution poorly describes their ultimate abilities to form α -helix when mixed with htt N-terminal fragments. Finally, it is likely that gross amphipathicity is not an adequate description of optimal hydrophobic topology for mixed oligomer formation and inhibition.

In spite of the imperfect correlation with α -helix parameters, overall – given the established structures of the htt^{NT} oligomers and the strong inhibitory activity of the D-versions of htt^{NT} – the data imply an important role for amphipathic helix formation in controlling the ability of peptides to inhibit the aggregation of htt N-terminal fragments.

Hybrid inhibitors

As shown in Figure 2, under most conditions htt^{NT} inhibitors are effective at delaying nucleation of amyloid formation in htt N-terminal fragment peptides, but cannot completely eliminate nucleation. This is likely because of the statistical probability that nucleation will at some point occur even within mixed oligomers, especially the sub-population of oligomers containing a relatively low percentage of inhibitor molecules. We hypothesized that nucleation inhibition might be enhanced if the inhibitor htt^{NT} peptide were fused to a powerful elongation inhibitor. We previously described such elongation inhibitors, in the form of mutated polyQ peptides that contain not only regularly spaced Pro-Gly residues, to dictate the points at which the chain can form β -turns, but also intervening Pro residues, to interfere with β -sheet formation in these β -hairpin structures^{23; 48}. We provided evidence that these peptides are reversible inhibitors of seeded polyQ elongation⁴⁸. For the present experiment, we fused such an inhibitor peptide, PGQ₉P^{1,2,3}, to htt^{NT} to produce a highly mutated htt N-terminal fragment analog (Table 1).

We found that a slight molar excess of this peptide with respect to htt^{NT}Q₃₀P₆K₂ inhibits aggregation of this htt N-terminal fragment with far greater potency than htt^{NT} alone (compare Fig. 8a, ●, to Fig. 2a, ◆). Pelletable aggregates containing up to 30% of the N-terminal fragments are formed within 50 hrs, but then no further aggregation takes place in this time frame. This transpires in parallel with the incorporation of about 20% of the inhibitor peptide htt^{NT}PGQ₉P^{1,2,3} over the same time frame (Fig. 8a, ●). Electron micrographs show that the aggregates produced in this co-incubation are almost entirely oligomeric even after 120 hrs (Fig. 4p).

Htt^{NT}PGQ₉P^{1,2,3} incubated alone generates very little pelletable aggregate in kinetics analysis (not shown), and only monomer and small aggregates in a dynamic light scattering (DLS) analysis at 3 hrs (Fig. 8b). Incubation of this peptide in isolation for 120 hrs shows a uniform population of regular oligomeric aggregates in the 10–30 nm range by EM (Fig. 4o), in good agreement with the 3 hr DLS measurement. Incubation of this peptide for 24 hrs followed by SEC gives two major peaks, one at the void (>700 KDa) corresponding to well-behaved aggregates and one at ~ 14 KDa, corresponding to the monomer (Fig. 8c). Collection of the void peak followed by incubation at 37 °C showed that this oligomeric fraction is moderately stable, not decaying for the first 24 hrs but diminishing after 72 hrs with concomitant regeneration of monomer (Fig. 8c inset). These data suggest that htt^{NT} fused to PGQ₉P^{1,2,3} produces a molecule that is about as capable of making oligomers as are polyQ-containing htt N-terminal fragments of similar size³², but which, in contrast to the unbroken polyQ versions, cannot progress to make amyloid fibrils. Interestingly, these isolated oligomers dissociate to monomer only slightly more slowly than the dissociation of isolated htt^{NT}Q₈K₂ oligomers (M. Jayaraman et al., Ms. submitted), consistent with a dominant role of htt^{NT} packing in determining oligomer stability.

Intrinsic fluorescence confirms the compatibility of the htt^{NT}PGQ₉P^{1,2,3} molecule with regular oligomer structure. Fluorescence of isolated aggregates from an incubated mixture of

htt^{NT}PGQ₉P^{1,2,3} (F17W) and htt^{NT}Q₃₀P₆K₂ (Fig. 5a, orange) yields a strong fluorescence spectrum (confirming the presence of the Pro-rich analog in the aggregates) with an emission maximum identical to that of homogeneous htt^{NT}Q₃₀P₆K₂ (F17W) oligomers (Fig. 5a, green). This suggests that, in contrast to simple htt^{NT} inhibitors, htt^{NT}PGQ₉P^{1,2,3} inhibitors co-assemble with htt^{NT}Q₃₀P₆K₂ peptides as a true structural analog of htt^{NT}Q₃₀P₆K₂.

Cell-penetrating versions of oligomer-targeted inhibitors

The ability of htt^{NT} and derivatives to slow or block nucleation of amyloid structure within oligomeric intermediates of htt N-terminal fragments suggests possible therapeutic applications. One means to deliver peptides into cells is by fusion to cell-penetrating peptide (CPP) sequences⁴⁹. We designed inhibitors in which the CPP sequence is placed on the C-terminus of the inhibitors, and chose as a CPP the artificial Lys₈ sequence⁵⁰, since Lys₈ merely extends the Lys₂ moiety we typically add to our peptides to improve kinetic solubility⁵¹ (Table 1). In the case of the htt^{NT} peptide, we also incorporated a Gly₅ spacer. We thus tested C-terminal Lys₈ extensions of both htt^{NT} (Fig. 9a) and htt^{NT}PGQ₉P^{1,2,3} (Fig. 9b). The results show that the inhibitory activity of the htt^{NT}-based molecules is not negatively affected by the C-terminal Lys₈ extension, and, at least in the case of htt^{NT} itself, appears to be somewhat improved by it (Fig. 9a). We also confirmed the ability of one of these peptides to readily enter mammalian cells by obtaining a Cys analog, htt^{NT}G₄CK₈, modifying it with Alexa633 (Methods), and adding the purified peptide to SHSY5Y cells in culture. After 10 mins incubation, followed by cell washes, the cells exhibited a strong fluorescence indicating cell-penetration (Fig. 9c). As previously found for other cell penetrating peptides, cell uptake was improved, and cytoplasmic localization facilitated, with the inclusion of 1-pyrenebutyric acid in the medium⁵².

DISCUSSION

The precise role of early, non-amyloid, oligomeric intermediates in amyloid assembly has long been debated. In different amyloid systems, evidence has been presented for their being either on-pathway^{53; 54} or off-pathway^{55; 56}. In the case of polyQ-containing htt N-terminal fragments, we have made a case for the initial α -helix rich oligomers being *both* on-pathway and off-pathway (Jayaraman et al., Ms. submitted). These roles are reflected in the current working model Fig. 10a).

Previously we reported that, for htt N-terminal fragment aggregation, a number of aggregate structural parameters, including Trp fluorescence quenching, ThT binding, polyQ accessibility, seeding capability, and fibrillar morphology all begin to change just at the time where aggregation kinetics dramatically increase³². In more recent work (Jayaraman et al., Ms. submitted), we provided evidence for an initial build-up of α -helix rich aggregates based on tetrameric building blocks in equilibrium with monomers. These early aggregates begin to accumulate β -structure in FTIR spectra contemporaneously with a fluorescence change associated with onset of amyloid formation. We interpreted these data to indicate the existence of a key structural conversion (i.e., nucleation) within the oligomer population that is required for onset of rapid fibril growth (Fig. 10a, structure 3). Since, in analogy to htt^{NT}PGQ₉P^{1,2,3} oligomers (Fig. 8c), isolated htt^{NT}Q_N oligomers readily dissociate to monomers (Jayaraman et al., Ms. submitted), we hypothesized that some oligomers never undergo the structural conversion of nucleation, instead serving as a reservoir for monomer release to sustain late-stage amyloid growth (M. Jayaraman et al., Ms. submitted). Thus, some α -helix rich oligomers may serve an on-pathway role, and some an off-pathway role (M. Jayaraman et al., Ms. submitted) (Fig. 10a). Since the htt^{NT} sequence alone undergoes a regular tetramerization and forms α -helix rich aggregates (M. Jayaraman, Ms. submitted), it is likely that self-associated α -helical htt^{NT} segments serve as the core of htt^{NT}Q_N.

oligomers. Interestingly, part of the htt^{NT} sequence remains in α -helix throughout nucleation and elongation (Jayaraman et al., Ms. submitted) and is even retained in the mature amyloid fibrils as a stable, water accessible structural element⁴³ (Jayaraman et al., Ms. submitted) (Fig. 10a).

In this paper we describe the characterization of two classes of inhibitors that interfere with the nucleation mechanism discussed above. One class of inhibitor includes molecules that are capable of co-assembling with polyQ-containing htt N-terminal fragments into mixed oligomers, resulting in a decreased local concentration of polyQ within these oligomers (Fig. 10b). The lower density of polyQ in these mixed oligomers decreases the frequency of interactions between polyQ segments and thereby reduces the effectiveness of either nucleus formation and/or nucleus elongation, and therefore the overall efficiency of nucleation. This class of inhibitors includes htt^{NT} and its short polyQ and Lys₈ extensions, as well as various analogs of htt^{NT}, including all-D htt^{NT}, retro-inverso htt^{NT}, and some htt^{NT} scrambled sequences. The other class of inhibitor consists of molecules that contain an htt^{NT} segment plus a mutated polyQ sequence designed so that, within the mixed oligomer (Fig. 10c), they can directly inhibit polyQ aggregate formation and elongation. This class is represented in this paper by the peptides htt^{NT}PGQ₉P^{1,2,3} and htt^{NT}PGQ₉P^{1,2,3}K₈. A number of small molecules have been identified that are capable of slowing huntingtin N-terminal fragment amyloid formation in vitro. One compound, (–)-epigallocatechin-3-gallate (EGCG), was shown to lead to increased the sizes of oligomeric intermediates in the process of reducing amyloid formation⁵⁷. Although details may be substantially different, it is interesting that enlarged oligomers accumulate in reactions inhibited by both htt^{NT}-related peptides and EGCG.

These htt^{NT}-related inhibitor peptides may have therapeutic applications, depending on the structural nature of the HD toxic species. Thus, if some form of mature amyloid aggregate is responsible for HD pathology, then peptides that inhibit the nucleation of amyloid structure should be protective. While delivery and stability of peptide drugs is always an issue, we show here that the inhibitory activity of htt^{NT} is maintained when the peptide is synthesized from D amino acids (expected to stabilize the peptide against proteases), and inhibitory activity is actually enhanced when the cell-penetrating peptide sequence Lys₈ is appended to htt^{NT}. On the other hand, if the toxic species is an oligomer¹⁰, then the inhibitors described in this paper might be counterproductive, since they actually function by stimulating the formation of mixed oligomers with extended lifetimes and restricted abilities to mature into amyloid. Interestingly, a molecule similar to htt^{NT}PGQ₉P^{1,2,3} has already been tested in a cell model. Subsequent to our reports of the aggregation properties of Pro-Gly interrupted polyQ molecules^{23; 48}, Poirier et al. expressed htt exon1 analogs containing very similar hypermutated polyQ sequences, including one resembling htt^{NT}PGQ₉P^{1,2,3}, in cultured cells and primary cortical neurons. Cells producing these proline/glycine polyQ mutants exhibited only minor levels of both visible aggregates and cytotoxicity⁵⁸. Our characterization in this paper of the aggregation properties of htt^{NT}PGQ₉P^{1,2,3} suggests that the protein expressed in the Poirier et al. experiments, while limiting the amount of accumulated amyloid, may have produced significant amounts of oligomers, such as those shown in Figure 3o. Considering the Poirier et al.⁵⁸ report in the context of the results described here, the simplest interpretation is that oligomers of htt N-terminal fragments are not toxic. However, it is possible that the hypermutated polyQ, while not diminishing oligomer formation, somehow diminishes oligomer toxicity (in ways other than by inhibiting amyloid formation). Based on our results, it would now be interesting to investigate cell models co-expressing a normal expanded polyQ htt N-terminal fragment along with an htt^{NT}PGQ₉P^{1,2,3}-like inhibitor.

Regardless of their prospects as therapeutics, the molecules described here are important in helping to validate the mechanism for htt N-terminal fragment aggregation nucleation (Fig.

10a) as well as support a recent, general model for amyloid formation by disordered peptides like A β and islet amyloid polypeptide⁵⁹. In their step-wise model, Abendini and Raleigh propose initial formation of oligomeric intermediates composed of peptides in which one segment is bundled into α -helix rich cores while another segment is left disordered. In a following step, the highly concentrated disordered segments interact to initiate amyloid structure formation. In their model, the initial α -helical segment later reshapes to become incorporated into the amyloid β -sheet. In contrast, in the case of htt fragments, the htt^{NT} segment appears to remain in α -helix in the mature amyloid fibril product⁴³ (Jayaraman et al., Ms. submitted). In this paper, we provide further support for this general mechanism by demonstrating the efficacy of inhibitor molecules designed to co-assemble into mixed α -helical oligomers.

For example, htt^{NT} and a variety of analogs appear to inhibit amyloid nucleation by efficiently co-assembling with htt N-terminal fragments into mixed oligomers. This leads to a reduction in the local concentration of polyQ elements on the surface of the oligomers, which reduces their ability to interact to form β -sheet rich nuclei for amyloid formation. Although a similar mechanism may be at work in the ability of C-terminal fragments of the A β molecule to make mixed oligomers with full length A β and to inhibit A β toxicity⁶⁰, detailed studies have not revealed any correlation of inhibitory activity with the ability to make α -helical oligomers^{61; 62}.

In this paper we also describe how htt^{NT} derivatives with an attached inhibitor of polyQ elongation, like htt^{NT}PGQ₉P^{1,2,3}, are particularly strong inhibitors of nucleation of amyloid structure in htt N-terminal fragments. These inhibitors most likely act by co-assembling with htt N-terminal fragments to make oligomers in which nucleation by surface-exposed polyQ elements is actively inhibited by the ability of the PGQ₉P^{1,2,3} element to interfere with polyQ amyloid formation. This makes this class of inhibitor conceptually identical to a recently described inhibitor of IAPP amyloid formation^{59; 63}. In that case, one or more proline residues were inserted into a portion of IAPP that did not disrupt its ability to make oligomers or co-oligomers, but did interfere with nucleation of amyloid formation^{59; 63}.

The structural details by which α -helix rich oligomers are held together remain to be elucidated. Feasible models for tetramer structure include parallel and anti-parallel helical bundles, as well as hemoglobin type folds in which α -helices are more orthogonally packed. Tetramers might assemble further into sedimentable oligomers either through the htt^{NT} segments, the polyQ segments, or both. However, since htt^{NT} alone forms tetramers, regular higher multimers, and sedimentable α -helical oligomers (M. Jayaraman et al., Ms. submitted), and since, as shown here, htt^{NT} itself co-assembles with htt^{NT}Q_N sequences and effectively inhibits amyloid nucleation, the simplest model consistent with the accumulated experimental data requires only htt^{NT} contacts for oligomer formation.

Besides these implications for the specificity of mixed oligomer formation, the scrambled peptide results make an additional point. The fact that a collection of scrambled sequences exhibits three different behaviors, i.e. (a) no effect, (b) amyloid formation, and (c) nucleation inhibition, points out the limitations of the common practice of constructing only a single scrambled sequence control as a test for specificity in the behavior of a bioactive peptide.

METHODS

Materials and general methods

Synthetic peptides were obtained crude and purified by reverse phase HPLC and confirmed by mass spectrometry as described previously⁶⁴. Most peptides were from the Keck

Biotechnology Center at Yale University; the scrambled htt^{NT} sequences were commissioned from GenScript, Inc. and Sigma, Inc. Acetonitrile, hexafluoroisopropanol (99.5%, spectrophotometric grade), and formic acid were from Acros Organics, and trifluoroacetic acid (99.5%, Sequanal Grade) from Pierce. Most peptides were disaggregated using a 1:1 mixture of HFIP and TFA, as described⁶⁴. The exceptions are the random sequences listed in Table 2, which were available in too low an amount for our normal disaggregation protocol. The scrambled sequence peptides were dissolved in pH 3 water and centrifuged at 386,000 g for two hrs in a table top ultracentrifuge to remove any aggregates. Htt^{NT} gave identical inhibition whether disaggregated by centrifugation only or by our standard TFA/HFIP protocol. Aggregation rates were determined by a sedimentation assay in which aliquots of ongoing reaction mixtures were centrifuged, and the amount of monomer remaining determined by analytical reverse phase HPLC of the supernatant⁶⁴. Some peptides contained a Phe17->Trp replacement in order to use Trp fluorescence to gauge the residue's solvent accessibility in aggregates. This replacement was previously shown to have only a very small effect, at best, on aggregation kinetics³². The isolation of aggregates for seeding and fluorescence analysis was conducted by centrifuging a reaction aliquot at $20817 \times g$ in an Eppendorf centrifuge at 4 ° C for 30 mins, washing the pellet 2–3 times with PBS, resuspending in buffer, and determining the aggregate concentration by an HPLC analysis of a formic acid dissolved aliquot, as described⁶⁴. Intrinsic tryptophan fluorescence of isolated aggregates was determined as described⁶⁵. DLS was performed on a Wyatt Dynapro microplate system using the built in software. Scrambled sequences were generated by using the randperm function in MATLAB. The input consisted of the wild type htt^{NT}Q sequence and the required number of scrambled versions was set at 50. The inbuilt randperm function was then used to rearrange the sequence randomly and generate 50 permutations. Theoretical helical propensities were calculated using the AGADIR program online (<http://agadir.crg.es/>). The conditions used for the calculations were pH 7.4, 298K and 0.1 ionic strength. Hydrophobic moments⁴⁷ were calculated by assuming entirely α -helical structure and analyzing the sequence using the Analysis module of the Heliquet program (<http://heliquet.ipmc.cns.fr>) with a window size of 18.

Electron microscopy

Aliquots of aggregation reaction mixtures were taken at different time points and visualized by electron microscopy. A 3 μ l sample was placed on a freshly glow-discharged carbon-coated grid, allowed to adsorb for 2 minutes, and washed with deionized water before staining with 2 μ l of 1% uranyl acetate and blotting. Grids were imaged on a Tecnai T12 microscope (FEI Co., Hillsboro, Oregon) operating at 120kV and 30,000x magnification, and equipped with an UltraScan 1000 CCD camera (Gatan, Pleasanton, California) with post-column magnification of 1.4x.

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy of various samples was performed using MB series spectrophotometer with PROTA software (ABB Bomem). Protein aggregates were harvested by centrifugation at 14,000 rpm and the pellet was washed 3 times with PBS. Spectra of re-suspended aggregates were recorded at 4 cm^{-1} resolution (400 scans at room temperature). Spectra were corrected for the residual buffer absorption by subtracting the buffer alone spectrum interactively until a flat baseline was obtained between 1700–1800 cm^{-1} . Second-derivative spectra for the Amide I region were calculated from the primary spectrum by using PROTA software.

Circular dichroism spectroscopy and spectral analysis

Far-UV CD measurements were performed on a JASCO J-810 spectropolarimeter using a 0.1 cm path length cuvette. Peptide samples were prepared by dissolving pure peptide in

acidic water (pH 3) and centrifuging at $435,680 \times g$ for 2 hrs, using the top 50% of the supernatant as the stock solution. All samples were prepared in 10mM Tris, pH 7.4 at concentrations between 76 μM – 150 μM . Far-UV CD spectra were collected at 1nm resolution with a scan rate of 100nm/min and averaged over four scans and corrected for the buffer signal. % helicity values were calculated using the CONTINLL program in the CDPRO package with the SP37A (ibasis 5) reference set.

Test for low molecular weight complex formation

A mixture of 28.9 μM htt^{NT}Q₃₀P₆K₂ (F17W) and 34.5 μM htt^{NT} in 10 mM sodium phosphate, 150 mM NaCl with 0.05% sodium azide, pH 7.4, was incubated at 37 °C for 3 hrs, then centrifuged (5000 rpm) and 200 μL of the supernatant injected onto a Superdex 75 10/200 column (GE Healthcare Life Sciences) equilibrated in the same buffer. 500 μL portions of the peak fractions were then analyzed on a reverse phase HPLC column on an Agilent 1100 LC-MS system.

Fluorescence labeling of cell penetrating peptide

Purified htt^{NT}G₄CK₈ was disaggregated⁶⁴ and the resulting dried film (~1.5mg) was resuspended in 1ml of deoxygenated labeling buffer (20mM HEPES pH 8.2, 5mM EDTA, 25mM tris(2-carboxyethyl)phosphine (TCEP; Pierce, Inc.)). The peptide solution was centrifuged 1 hr at 100,000 g and the concentration was checked by HPLC. A 5:1 molar excess of Alexa Fluor® 633 C₅-maleimide (Invitrogen/Molecular Probes) was dissolved in 50 μL of labeling buffer and added drop-wise to the peptide solution. The labeling reaction, incubated with constant stirring at 23 °C, was ~50% complete after 1 hr, and complete after overnight reaction, by analytical HPLC. Peptide components were separated from buffer and excess labeling reagent on a D-Salt™ Polyacrylamide Desalting Column (Pierce) equilibrated in 20mM HEPES pH 8.2, 5mM EDTA buffer. Fractions (0.5 ml) were collected and checked by HPLC-mass spectrometry to confirm the identity and purity of the Alexa labeled peptide. Peptide fractions were pooled and aliquots containing desired amounts of peptide were lyophilized and stored at –80 °C.

Cell uptake of fluorescent cell penetrating peptide

SHSY5Y neuroblastoma cells were seeded at 5×10^5 cells per well in a 96 wells plate and cultured for 24hr in complete growth medium (DMEM (Cellgro) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine (GIBCO) and Penicillin-Streptomycin (HyClone)). A frozen sample of the labeled inhibitor peptide (see above) was disaggregated overnight in TFA-HFIP and the solvents were evaporated under a stream of argon. The peptide film was further dried under vacuum for 1 hour and resuspended in PBS⁶⁴. The peptide concentration of the PBS solution was calculated from the absorbance at 633nm using the extinction coefficient reported by the manufacturer. Prior to the experiment, cells were washed with warm PBS and incubated with 50 μL of 100 μM 1-pyrenebutyric acid (Sigma) in PBS for 5 mins at 37°C after which 50 μL of 10 μM inhibitor peptide was added to achieve a final concentration of 5 μM . After 10 minutes of incubation at 37°C, cells were washed four times with warm PBS and imaged using an Olympus IX71 inverted fluorescent microscope equipped with a cy5 filter.

Curve fitting

The aggregation kinetics of all the control substrate reactions were fit to a three-parameter exponential decay function in keeping with our current understanding of the aggregation of these peptides. Similarly, the aggregation kinetics of the substrates in the presence of the htt^{NT} and its analogs were fitted with a three parameter sigmoidal function in order to describe the lag-phase that is observed in such inhibition. On the other hand, in the

inhibition reaction with the htt^{NT}PGQ₉P^{1,2,3} inhibitor, in which the only detectable aggregate formed is an apparently stable population of mixed oligomers, the aggregation kinetics of the substrate were fit to a three-parameter exponential. It should be noted that curve fits have been conducted here both to visually organize the data to improve accessibility, and to allow objective estimation of $t_{1/2}$ times of aggregation, but not to support any particular mechanistic hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Kinetic effects of htt^{NT} on aggregation of polyQ-containing peptides by the sedimentation assay. (a) Aggregation of 23.3 μM htt^{NT}Q₃₇P₁₀K₂ (●); 25.2 μM SWQ₃₇P₁₀K₂ (▲); 25.6 μM K₂Q₃₅P₁₀K₂ (▲); 7.5 μM K₂Q₄₁K₂ alone (◆); and 7.0 μM K₂Q₄₁K₂ plus 17.4 μM htt^{NT} (■). (b) Aggregation of 18.8 μM of the peptide ESLKSF-Q35-PPPSKETAAAKFERQHMDs incubated alone (●) or with 29.6 μM htt^{NT} (●).

Figure 2.

Htt^{NT} inhibition of aggregation. (a, b) Incubation of 9 μ M htt^{NT}Q₃₀P₆K₂ with different molar ratios of htt^{NT} to htt^{NT}Q₃₀P₆K₂: 0.05:1 ($\circ$); 0.16:1 ($\blacktriangle$); 0.43:1 ($\triangle$); 0.89:1 ($\blacklozenge$). (a) Soluble htt^{NT}Q₃₀P₆K₂ after centrifugation; control incubation of htt^{NT}Q₃₀P₆K₂ without inhibitor ($\bullet$); (b) Soluble htt^{NT} inhibitor after centrifugation; control incubation of htt^{NT} alone ($\bullet$); (c) Effect of higher ratios and multiple additions of htt^{NT} on htt^{NT}Q₃₀P₆K₂ aggregation: 7.0 μ M htt^{NT}Q₃₀P₆K₂ alone ($\bullet$); 5.33 μ M htt^{NT}Q₃₀P₆K₂ with either 7.5 μ M htt^{NT} (i.e., $\sim$ 1.4 fold molar excess) ($\circ$) or 13.3 μ M htt^{NT} (i.e., $\sim$ 2.5 fold molar excess) ($\triangle$) added at t = 0; 5.33 μ M htt^{NT}Q₃₀P₆K₂ plus 7.5 μ M htt^{NT} reaction in which fresh, additional aliquots of htt^{NT} were added at 7 hrs (11.2 μ M), 24 hrs (7.0 μ M), 31 hrs (7.3 μ M), and 68 hrs (6.4 μ M) ($\blacksquare$). Peptides were incubated at 37 $^{\circ}$ C in PBS, with aggregation monitored by the sedimentation assay.

Figure 3.

The SEC profile of an incubated mixture of htt^{NT}Q₃₀P₆K₂ (F17W) and htt^{NT} was analyzed by LC-MS. The peak eluting at ~ 32 mins gave the HPLC profile shown in part B, and the mass spectrum at the intensity peak (inset) gave a mass of 6697 Da consistent with htt^{NT}Q₃₀P₆K₂ (F17W). The peak eluting at ~49 mins gave the HPLC profile shown in Part C, and the mass spectrum at the intensity peak (inset) gave a mass of 1972.8 Da, consistent with htt^{NT}. The absence of a SEC peak containing both peptides indicates that there is no significant low molecular weight complex formation under these conditions.

Figure 4.

Electron micrographs of aggregates of htt N-terminal fragments. Aggregates from Htt^{NT}Q₃₀P₆K₂ alone incubated in PBS at 37 °C and sampled at 0 hrs (a), 15 min (b), 5.5 hrs (c), 24 hrs (d), 48 hrs (e), and 100 hrs (f). Htt^{NT}Q₃₀P₆ incubated with htt^{NT} in 1:1 ratio in PBS, 37 °C and sampled at 3 hrs (g, h), 5.5 hrs (i, j), 24 hrs (k, l), 48 hrs (m) and 72 hrs (n). htt^{NT}PGQ₉P^{1,2,3} incubated alone for 120 hrs (o), htt^{NT}Q₃₀P₆K₂ and htt^{NT}PGQ₉P^{1,2,3} co-incubated in 1:4 ratio in PBS, 37 °C for 120 hrs (p). Scale bar represents 50 nm.

Figure 5.

Spectroscopic probes of aggregate structure. (a) Tryptophan fluorescence spectra of isolated and resuspended aggregates from the following reactions: monomeric $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_6\text{K}_2$ (F17W), $\lambda_{\text{em}} = 355$ nm (black); aggregated $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_6\text{K}_2$ (F17W) alone, $\lambda_{\text{em}} = 347.5$ nm, (green); htt^{NT} plus $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_6\text{K}_2$ (F17W), $\lambda_{\text{em}} = 349$ nm, (magenta); $\text{htt}^{\text{NT}}\text{Q}_3$ (F17W) inhibitor plus $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_6\text{K}_2$, $\lambda_{\text{em}} = 337$ nm, (blue); $\text{htt}^{\text{NT}}\text{PGQ}_9^{\text{P1,2,3}}$ (F17W) plus $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_6\text{K}_2$, $\lambda_{\text{em}} = 347.5$ nm, (orange). (b) Time course of the Trp fluorescence peak shift in isolated aggregates when the $\text{htt}^{\text{NT}}\text{Q}_3$ (F17W) inhibitor is incubated with $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_6\text{K}_2$ at a 1.1:1 ratio; (c) Second derivative FTIR spectra of aggregates isolated at about 100 hrs from incubation reactions of htt^{NT} (black), $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_6\text{K}_2$ (red), and a reaction with a 1:1 mixture of htt_{NT} and $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_6\text{K}_2$ (blue); (d) primary FTIR spectra of aggregates generated upon incubation of either all-L htt^{NT} (blue), all-D htt^{NT} (red), and retro-inverso htt^{NT} (green).

Figure 6.

Abilities of approximately equimolar htt^{NT} analogs to inhibit aggregation of htt N-terminal fragments in PBS at 37 °C, as determined by a sedimentation assay. (a) inhibition of 7.6 μM $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_{10}\text{K}_2$ aggregation by all-L htt^{NT} (15 μM), all-D htt^{NT} (11.1 μM) and retroinverso- htt^{NT} (9.5 μM) (b, c) inhibition of 5–10 μM $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_6\text{K}_2$ aggregation by 1:1 molar amounts of wild type and scrambled $\text{htt}^{\text{NT}}\text{Q}$ sequences.

Figure 7.

Scatterplot analyses of inhibitory activities of wild type htt^{NT} and scrambled peptides (Table 2). (a) aggregation inhibition vs. AGADIR α -helix propensity (linear fit; $R^2 = 0.80$); (b) aggregation inhibition vs. hydrophobic moment (μH) score (exponential fit; $R^2 = 0.88$); (c) aggregation inhibition vs. normalized μH values determined by multiplying the hydrophobic moment times the measured % α -helix as determined by CD spectroscopy (three parameter exponential fit; $R^2 = 0.70$) (see Table 2). In each plot the position of the htt^{NT} wild type sequence is in red.

Figure 8.

Properties of htt^{NT}PGQ₉P^{1,2,3}. (a) inhibition htt^{NT}Q₃₀P₆K₂ aggregation by equimolar htt^{NT}PGQ₉P^{1,2,3}; (b) DLS of htt^{NT}PGQ₉P^{1,2,3} incubated alone for three hrs; (c) SEC analysis of oligomer assembly and disassembly of htt^{NT}PGQ₉P^{1,2,3}. 300 μ M peptide was incubated for 24 hrs, then separated by SEC in PBS running buffer (dashed line). The oligomer peak (~18 mins) was collected and incubated at 37°C, then analyzed at the times shown. Between 44 and 72 hrs a substantial portion of oligomer (~18 mins) dissociates to monomer (~42 mins).

Figure 9.

Properties of cell penetrating peptide versions of inhibitors. (a) Inhibition of $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_6\text{K}_2$ aggregation by $\text{htt}^{\text{NT}}\text{G}_5\text{K}_8$ and controls; (b) Inhibition of $\text{htt}^{\text{NT}}\text{Q}_{30}\text{P}_{10}\text{K}_2$ aggregation by $\text{htt}^{\text{NT}}\text{PGQ}_9\text{P}_{1,2,3}\text{K}_8$ and controls; (c) Cytosolic delivery of $\text{htt}^{\text{NT}}\text{G}_4\text{CK}_8$ -Alexa633 into SHSY5Y cells (Methods). After 10 mins exposure to peptide plus 1-pyrenebutyric acid, cells were washed extensively with PBS and imaged.

Figure 10.

α -Helix rich molecules in htt N-terminal fragment aggregation and its inhibition. (a) A model for nucleated growth in htt N-terminal fragment aggregation (modified from Jayaraman et al., Ms. submitted). When the htt N-terminal fragment monomer (a1: htt^{NT}, green; polyQ, orange; Pro-rich domain, black) assembles into the oligomeric intermediate (a2), the htt^{NT} segment takes on α -helix structure whose packing with other htt^{NT} segments into a helical bundle stabilizes the oligomer. As a consequence, the disordered polyQ chains are brought together closely in space within the oligomer, which lowers the barrier to formation of nuclei with amyloid structural elements (a3, polyQ extended chain, red). Monomers present at this stage facilitate elongation into growing amyloid fibrils (a4, a5, a6). In the final stages of amyloid growth, as the monomer pool is depleted, oligomers that have not undergone nucleation dissociate (a2 $\rightarrow$ a1) to generate more monomers to support fibril elongation. (b) structural model for the inhibition complex (oligomer) between htt^{NT} (blue cylinders) and an htt N-terminal fragment; (c) structural model for the inhibition complex between an htt N-terminal fragment and a C-terminally extended htt^{NT} inhibitor, such as htt^{NT}PGQ₉P^{1,2,3} or htt^{NT}PGQ₉P^{1,2,3}K₈ (blue cylinders = htt^{NT} helices; blue lines = PGQ₉ sequence; red circles = additional Pro residues (see Table 1)).

Table 1

Peptide sequences studied

Name	Sequence
Htt ^{NT}	MATLEKLMKA FESLKSF
Htt ^{NT} Q ₃ (F17W)	MATLEKLMKA FESLKSQQQ
Htt ^{NT} G ₅ K ₈	MATLEKLMKA FESLKSFGGG GGGGGGGGGG
Htt ^{NT} G ₄ CK ₈	MATLEKLMKA FESLKSFGGG GGGGGGGGGG
Htt ^{NT} Q ₃₀ P ₆ K ₂	MATLEKLMKA FESLKSQQQ QQQQQQQQQQ QQQQQQQQQQ QQQQQQQPPP PPPKK
Htt ^{NT} Q ₃₀ P ₆ K ₂ (F17W)	MATLEKLMKA FESLKSQQQ QQQQQQQQQQ QQQQQQQQQQ QQQQQQQPPP PPPKK
Htt ^{NT} Q ₃₀ P ₁₀ K ₂	MATLEKLMKA FESLKSQQQ QQQQQQQQQQ QQQQQQQQQQ QQQQQQQPPP PPPPPPKK
Htt ^{NT} Q ₃₇ P ₁₀ K ₂	MATLEKLMKA FESLKSQQQ QQQQQQQQQQ QQQQQQQQQQ QQQQQQQPPP PPPPPPKK
SWQ ₃₇ P ₁₀ K ₂	SWQQQQQQQQ QQQQQQQQQQ QQQQQQQQQQ QQQQQQQPPP PPPPPPKK
K ₂ Q ₃₅ P ₁₀ K ₂	KKQQQQQQQQ QQQQQQQQQQ QQQQQQQQQQ QQQQQQQPPP PPPPPPKK
K ₂ Q ₄₁ K ₂	KKQQQQQQQQ QQQQQQQQQQ QQQQQQQQQQ QQQQQQQQQQ QQQKK
PGQ ₉ P ^{1,2,3}	KKQQQQQQQQ QPGQQQQPQQ QPQQQQQPQQ QQQPGQQQQQ QQQQKK
Htt ^{NT} PGQ ₉ P ^{1,2,3}	MATLEKLMKA FESLKSQQQ QPQQQQPQQ QPQQQQPQQ QQQPQQQQPG QQQQQQQQK K
Htt ^{NT} PGQ ₉ P ^{1,2,3} (F17W)	MATLEKLMKA FESLKSQQQ QPQQQQPQQ QPQQQQPQQ QQQPQQQQPG QQQQQQQQK K
Htt ^{NT} PGQ ₉ P ^{1,2,3} K ₈	MATLEKLMKA FESLKSQQQ QPQQQQPQQ QPQQQQPQQ QQQPQQQQPG QQQQQQQQK KKKKKKK

Table 2

Sequences and properties of scrambled htt^{NT} sequence peptides^a

Peptide Number	Sequence	AGADIR score ^b	% helix, CD ^c	Hydrophob. moment (μH) ^d	t _{1/2} ^e
WT	MATLEKLMKAFESLKSF	4.27	8.7	0.398	90
2	MLSLKESAKMFFATKELQ	0.49	4.3	0.118	20
3	KQFTLEMAFLSKALSEMK	1.74	8.7	0.342	40
4	KLAFMLKQAELESSEKTFM	0.60	5.3	0.154	25
5	FAKFASEKKLESMTMLQ	0.56	3.5	0.092	18
6	MLTFAEFKSMELKSQLAK	1.12	0.0	0.137	20
7	ASMFEAQLSKEKKMFTLL	1.60	8.7	0.335	60
8	ELLAKSEQAQKSMMLFTFMK	0.59	3.9	0.305	35
9	TKFSFALLAQKEMLKME	1.29	6.2	0.203	25
11	ASSQKKMKEMLAFFTLEL	2.97	5.8	0.287	80
12	MFSKMAKSLFLAEKTQE	1.10	3.7	0.414	50
-	No addition	-			20

^a Scrambled sequence peptides, plus wild type htt^{NT}, investigated for ability to inhibit aggregation of htt^{NT}Q30P6K2 when present in 1:1 stoichiometry. Not shown are peptides that exhibited poor synthetic yields, co-migration with htt^{NT}Q30P6K2 in analytical HPLC, or facile amyloid formation when incubated alone in PBS at 37 °C.

^{b,c,d,e} See Methods.