INTRODUCTION

On the molecular level Alzheimer’s disease is characterized by proteinaceous aggregates. Extracellular polymers consist largely of polymerized Aβ-peptide [1]; those inside the cell contain polymerized microtubule-associated protein tau (for review [2, 3]). Studies on other amyloid-based neurodegenerative diseases have gathered evidence that insoluble aggregates or their oligomeric precursors are responsible for degeneration which leads to the impairment of cell functions typical for the disease [4-6].

The aggregates formed by tau protein exhibit a helical appearance and are called Paired Helical Filaments (PHFs) which tend to coalesce into larger structures, the neurofibrillary tangles. The distribution of the neurofibrillary changes correlates well with the development of the neurological symptoms and is used for the staging of Alzheimer’s disease [7]. This staging is part of the guidelines for post mortem diagnosis [8]. The Braak staging is based on the appearance of tau in an abnormal state of phosphorylation and aggregation. Hyperphosphorylation in general is believed to be a characteristic feature of tau in Alzheimer’s [9] but it is unclear whether phosphorylation is the cause or consequence of neuronal degeneration. For example, phosphorylation at KXGS motifs within the repeat domain of tau is known to decrease the affinity of tau towards microtubules [10], thereby increasing the concentration of unbound tau in the cytoplasm and hence the concentration-dependent aggregation [11-13]. On the other hand, the same kind of phosphorylation inhibits filament formation in in vitro experiments [14]. Besides phosphorylation, tau in Alzheimer’s exhibits further posttranslational changes (e.g. glycation or transglutamylation [15-17] and caspase cleavage, [18, 19]. Truncation outside the microtubule binding repeats leads to fragments with a higher tendency to polymerise than full length isoforms [20-22].

In other neurodegenerative diseases like Parkinson’s and Huntington’s disease it is reported that intracellular aggregates cause cytotoxic effects (for reviews see [23, 24]). In Parkinson’s disease the oligomeric structures are shown to influence the stability of the cell membrane [4]. In Huntington’s disease aggregated protein can be found in the nucleus [25], possibly affecting gene transcription. In a mouse model of Huntington’s disease it was shown that disaggregation of polymers leads to a prolonged life-time [26]. Consistent with this, measures which reduce the concentration of oligomers and aggregates alleviate the diseases [26-29].

Tau is intrinsically unstructured and highly soluble [30], yet it can aggregate into insoluble polymers. The soluble form of tau was analyzed by different spectroscopic techniques (CD, FTIR-spectroscopy, small angle X-ray scattering, gel filtration and limited proteolysis [11, 31, 32], all proving a mostly random coil conformation. However, the tau sequence contains certain motifs that appear to undergo a conformational change towards β-sheet structure. This can drive the protein into filaments that are indistinguishable from those of Alzheimer’s brain. Since the intracellular aggregation of tau in AD correlates with the clinical progression of the disease it seemed likely that inhibition or even reversal of the tau aggregation would protect or rescue the affected neurons. In order to test this hypothesis we began to identify inhibitors of tau aggregation. Here we summarize some procedures which were used to screen for compounds capable to inhibit tau aggregation.

Screening for Inhibitors of Tau Polymerization

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Abstract: The histopathological diagnosis of Alzheimer’s disease relies on two kinds of proteinaceous aggregates: the extracellular plaques built from filaments of the Aβ-peptide and the intracellular tangles consisting of tau polymerized into Paired Helical Filaments (PHFs). The order of aggregation events is still under debate, but it is well accepted that tau-related changes have an important impact on the viability of neurons. In neurons, early morphological changes are seen in axons which begin to loose and retract synapses. This process is accompanied by an increase of aggregated tau protein. Thus the prevention of tau aggregation seems to be a valuable target for therapy of Alzheimer’s disease. Here we present a screening procedure by which we identified inhibitors of tau polymerization. In the primary screen we used a thioflavin-S based assay which detects PHF formation in solution. These initial hits were further analyzed for their capacity to depolymerize preformed PHFs. These results were confirmed by several secondary assays (tryptophan fluorescence, pellet-based assay which detects PHF formation in solution. These initial hits were further analyzed for their capacity to depolymerize preformed PHFs. This staging is part of the guidelines for post mortem diagnosis [8]. The Braak staging is based on the appearance of tau in an abnormal state of phosphorylation and aggregation. Hyperphosphorylation in general is believed to be a characteristic feature of tau in Alzheimer’s [9] but it is unclear whether phosphorylation is the cause or consequence of neuronal degeneration. For example, phosphorylation at KXGS motifs within the repeat domain of tau is known to decrease the affinity of tau towards microtubules [10], thereby increasing the concentration of unbound tau in the cytoplasm and hence the concentration-dependent aggregation [11-13]. On the other hand, the same kind of phosphorylation inhibits filament formation in in vitro experiments [14]. Besides phosphorylation, tau in Alzheimer’s...
MATERIALS AND METHODS

Chemicals and Proteins

Heparin and thioflavin-S were obtained from Sigma. Tau proteins were expressed in E. coli and purified as described elsewhere [33].

PHF Assembly

Assembly of PHFs from tau protein (K19, 10 µM) was performed at 37 °C in the presence of polyanions (heparin; 5 µM) in 50 mM NH₄Ac, pH 6.8 as described elsewhere [32]. For screening the reaction was set up in 384 well microtiter plates.

Thioflavin-S Assay

PHF formation was monitored by thioflavin-S fluorescence assay as described [22]. Briefly, thioflavin-S was added to the aliquot of a polymerization reaction to a final concentration of 20 µM and the signal was measured using a fluorimeter (type Ascent; Labsystems with excitation at 440 nm and emission at 521 nm).

Electron Microscopy

Protein solutions diluted to 0.1-10 µM were placed on 600-mesh carbon-coated copper grids for 1 min and negatively stained with 2 % uranyl acetate for 45 sec. The specimens were examined in a Philips CM12 electron microscope at 100 kV.

Tryptophan Fluorescence Spectroscopy

The tryptophan fluorescence assay was performed as described in more details elsewhere [34]. Briefly, 10 µM of protein were analyzed in a Spex Fluoromax spectrophotometer (Polytec, Waldbronn, Germany), using 3 mm × 3 mm micro cuvettes from Hellma (Mühlheim, Germany) with 20 µl sample volumes. Emission spectra were measured from 300 to 450 nm after excitation at 290 nm.

Microtubule Assembly

Microtubule assembly was analyzed by light scattering at 350 nm in a spectrophotometer (Safire, Tecan, Crailsheim, Germany). Tau protein (5 µM) was mixed with tubulin dimer (30 µM) and GTP (1 mM) at 4°C in polymerization buffer (100 mM Na-PIPES pH 6.9, 1 mM EGTA, 1 mM MgSO₄, 1 mM DTT) in a final volume of 40 µl. After rapid mixing, the analysis was performed in wells of a 384 well microtiter plate. The assembly was started by incubation at 37°C. From the curve three parameters were extracted: the maximum turbidity at steady state, the rate of assembly, and the lag time between the temperature jump and the start of the turbidity rise.

Filter Trapping Assay

The filter trapping was adapted to tau aggregates from an assay used in detection of Huntingtin aggregates [35]. Aggregates of tau were trapped by filtration through a PVDF-membrane (pore diameter 0.45 µm, Schleicher and Schuell, Düren, Germany) adapted to a 96-well dot blot apparatus. The membrane was washed three times with PBS before usage and the sample was applied as a dilution in PBS. After filtration the membrane was blocked with milk powder (5 %) and the polyclonal tau antibody K9JA was used as primary antibody. Detection was performed by an anti-rabbit antibody conjugated with horseradish peroxidase (Dako, Hamburg, Germany) using the ECL-system (Amersham Pharma- cia) and the digital gel documentation system Fuji film LAS 3000 (Raytest, Straubenhardt, Germany). Quantification of the signals was performed with AIDA-software package (Raytest, Straubenhardt, Germany).

RESULTS

For the primary screen we decided to use K19, a fragment of tau containing three repeats (Fig. 1). It was known that this construct polymerizes in the presence of heparin within a reasonable time scale [22]. The polymerization characteristics of K19 are influenced by the single cystein at position 322. Oxidation of this residue yields dimers which
promote polymerization [21, 30, 36]. In contrast, tau fragments or isoforms containing two cysteins tend to form intramolecular disulfide bridges that are inhibitory for PHF formation [30]. By introducing a single tryptophan at position 310 we were able to analyze the effect of compounds on PHF formation or stability by using tryptophan fluorescence (see Fig. 4), which is sensitive to the local environment and therefore monitors PHF assembly [34].

In the primary screen we analyzed the impact of compounds on PHF formation. K19 at 10 µM was incubated with 60 µM of compound and PHF aggregation was induced by the addition of heparin (5 µM). After incubation at 37°C for 48 h the readout was performed by the thioflavin-S assay (Fig. 3). In controls the influence of the compounds on thioflavin-S fluorescence was analyzed. Compounds that affected thioflavin-S fluorescence were excluded from further analysis. Hits were defined by a reduction of aggregation by 90% compared to the control without any compound. About 0.63% of the compound library fulfilled this criterion.

In a secondary screen (see Fig. 2) we tested these hits for their capacity to depolymerise preformed PHFs. In this assay we incubated preformed filaments of K19 (10 µM) in the presence or absence of compounds and determined the amount of remaining PHFs by thioflavin-S fluorescence. Hits were defined as a decrease of thioflavin-S fluorescence by at least 80% of the control. About 0.04% of the library passed this criterion. These compounds were retested with independent measurements like tryptophan fluorescence (see Fig. 4), filter trapping assay (Fig. 5A), pelleting assay (Fig. 5B), electron microscopy (Fig. 6) and tau-microtubule binding assay (Fig. 7).

The thioflavin-S assay allows a fast and reliable measurement of PHF formation by tau protein and can be implemented into a medium or high throughput screening procedure. The principle of the thioflavin-S assay relies on the specific binding of thioflavin-S to the aggregated tau protein which leads to a strong increase of fluorescence emission (Fig. 3B). An example of the results from 80 compounds on one plate is given in Fig. 3C. The thioflavin-S emission is plotted as per cent of aggregation after normalization to the control. The black bars represent inhibition higher than 80%. In this plate thirteen compounds yield inhibition higher than 80% and three show inhibition of more than 90%. There are two compounds which yielded higher thioflavin-S fluorescence than the unaffected control, indicating that they might have stimulated aggregation or influenced thioflavin-S fluorescence independently of the polymerized protein.

The exchange of the tyrosine at position 310 into tryptophan enables the monitoring of PHF formation by tryptophan fluorescence as described elsewhere [34]. The emission spectrum of soluble tau protein exhibits a maximum at about 355 nm (solid curve in Fig. 4A) and undergoes a blue shift upon tau polymerization towards 340 nm (dotted curve in Fig. 4A). PHFs can be dissolved in the presence of 2 M GuHCl which causes the maximum to shift back to 355 nm (Fig. 4C, #3, 4). In this example (solid curve Fig. 4B) an inhibitor found in the screening was used to reduce the polymerization of K19, leading to an emission maximum of 352 nm, which corresponds to ~84% inhibition of polymerization. By the same method the stability of preformed K19-PHF can be analyzed in the presence of inhibitors. In the example of (Fig. 4B), the dotted curve represents the emission spectrum after partial dissolution of PHFs. The emission maximum of 348 nm corresponds to a depolymerization of about 54% (data summarized in Fig. 4C).

To check the results two more independent types of assay were applied which both make use of the change of the size...
Thioflavin-S fluorescence was used to measure the aggregation of tau into PHFs. The fluorescence maximum is shifted in the excitation scan (A) and the increase of emission at 480 nm (B) is proportional to the extent of PHF aggregation. The evaluation of a screening plate is shown (C). The extent of aggregation is shown in per cent of the aggregation of the untreated control and is colour coded (100-41%: white; 40-0%: black). (D) Aggregation of tau construct K19 (10 µM) vs. inhibitor concentration (1 pM-200 µM). The extent of aggregation was measured by thioflavin-S fluorescence assay and the degree of inhibition was plotted as percentage of control. The fits were calculated as four parameter logistic curves. For disassembly experiments (E) tau construct K19 (10 µM) was first aggregated into PHFs and the polymers were separated from the soluble tau by centrifugation at 100,000 g for 1 h. The pellet was redissolved and then exposed to an inhibitor overnight at 37°C at the indicated concentrations (range 1 pM-200 µM). Half-maximal inhibition occurs at 9.0 µM and half-maximal disassembly at 8.4 µM, respectively.

The wavelength of the emission maximum after the assembly reaction indicates the extent of polymerization. The negative control (soluble protein) exhibits an emission maximum around 355 nm (A, solid line; C, entry #1), whereas the positive control (aggregated protein without compound) shows an emission maximum at 340 nm (A, dotted line; C, #2). As a control, soluble and polymerized proteins exhibit the same emission maximum around 355 nm after denaturation and disassembly by GuHCL (C, #3 and 4). The emission maxima of the samples treated with inhibitor lies near 352 nm, illustrating that PHF formation is effectively inhibited (compare B, solid line and C, #5). But the inhibitor can disaggregate PHFs only to an extent of about 50% under these conditions (B, dotted line and C, #6).
10 µM). The other samples represent htau23 polymerized in the presence of inhibitors showing different potencies. In the second size-dependent approach the polymerization sample is centrifuged, and subsequently pellet and supernatant are analyzed by SDS-Page and densitometry (Fig. 5B). In the examples shown here the polymerization of K19 (10 µM) was analyzed in the presence of a compound at increasing concentrations (1-60 µM). At low concentrations of compound most of the protein is found in the pellet fraction due to aggregation, but at higher concentrations the majority of the protein remains soluble and appears in the supernatant.

DISCUSSION

In this report we describe some approaches to identify inhibitors of the aggregation of tau protein into Alzheimer PHFs. Several methods were used to confirm the results which at the same time yielded interesting and novel evidence on aspects of PHF assembly and stability. The fluorescent dye thioflavin-S binds to filamentous structures which contain β-sheet structure and is used for detection of a variety of different amyloids in different applications [38-45]. The structural basis of thioflavin-S binding is poorly understood, for example it is unknown which minimal length of a filament is required for binding. This may explain the variation of the thioflavin-S signal in different assembly conditions at early stages of assembly [36, 46]. The assessment of the impact of compounds on tau polymerization by thioflavin-S is therefore restricted to filaments with some minimum length and becomes unreliable for small oligomers. Nevertheless, this method is reliable for the quantification of tau PHF formation, particularly in conditions where assembly is induced by polyanionic cofactors [22]. A similar need for short filaments holds for electron microscopy and the filter trapping assay, which both require aggregates in the size of at least 400 kDa (determined by gel filtration) or particles with a diameter of 10 nm for detection. In contrast, the tryptophan fluorescence assay reports changes in the molecular surroundings of the reporter tryptophan which is sensitive to the state of aggregation [34].

This measurement is independent of the length of a filament and can detect changes on the level of 4-8 tau molecules interacting in a PHF-like conformation. It may therefore be more appropriate when information is sought on the presence of oligomeric species. One unexpected outcome of the Trp fluorescence assay was that PHFs are surprisingly unstable when formed initially and can therefore disrupted in relatively mild conditions, in contrast to the highly crosslinked and stable filaments isolated from late-stage Alzheimer brains [47]. The differences in sensitivity of the methods may explain why the IC₅₀ values measured by the thioflavin-S are lower than a comparable analysis by the tryptophan fluorescence assay. By a combination of these techniques we found a group of anthraquinones which are capable to inhibit and reverse PHF assembly [48]. A further well-established method is that of UV light scattering which is capable of sensing both oligomeric and polymeric species. It is commonly applied to microtubule assembly studies (Fig. 7) and has been successfully used to study PHF assembly [49].

The process of tau polymerization follows the route of a nucleation-dependent polymerization ([11]; cf. Fig. 8). A prerequisite for the formation of the nucleus is a conformational transition of short sequence motifs in the repeat domain of tau from random coil to β-structure [32]. This places tau protein in line with other amyloidogenic proteins whose aggregation is based on the formation of β-structure. One feature which makes the aggregation of tau unusual is that only a minor fraction of the protein appears to be involved in the aggregation, while the protein as a whole is highly soluble and thus resistant to aggregation. This has made it difficult to identify the principle of aggregation and the protein domains involved in it. Following a general model of amy-
Fig. (6). Electron microscopy of PHF disassembly.
PHFs were assembled from K19 (10 µM) in the presence of 2.5 µM heparin in 50 mM NH₄Ac buffer pH 6.8 at 37 °C over night. After assembly the inhibitor was added at 1 to 200 µM, and the degree of disassembly was monitored by electron microscopy. The examples show breakdown products after overnight incubation with inhibitors. Bar = 100 nm.

Fig. (7). Microtubule assembly. 30 µM tubulin dimer was incubated in a microtiter plate at 37°C in the absence and presence of htau40 (10 µM) and 60 µM Congo red. Absorbance was taken at 350 nm and plotted vs. time. Tubulin alone does not assemble (dash-dotted line), tubulin with tau assembles rapidly (dashed), Congo red reduces the assembly by ~30% (solid line).

Fig. (8). Model of PHF aggregation.
The diagram illustrates the formation of PHFs from tau protein (grey and black line) in a kinetic scheme: Starting from the soluble protein (left) a nucleus is formed (top right) which can grow to a filamentos structure (bottom). The repeats of tau undergo conformational change into β-sheet structure (black arrows), whereas the rest of the sequence (in grey) remains unstructured. Different classes of binding sites of compounds are shown by white stars. The PHF is miniaturized in order to show the dimension of the filament. Inhibitors could interfere with assembly by interacting directly with the PHF core, or by influencing the conformation of tau outside the core.
three possible mechanisms of interaction: firstly within the β-forming hexapeptides, which may block the addition of more β-strands or even destabilize the β-sheet and reverse the nucleus into soluble protein. An example analogous to this case is the introduction of a proline into the hexapeptide motifs by mutagenesis which blocks aggregation very efficiently [32]. Secondly an inhibitor might block the elongation, so that polymerization would lead to large amounts of oligomeric nuclei unable to grow further. This can be achieved by either blocking the binding site of the protein or of a cofactor like heparin. Thirdly the compounds could interact with sequences outside the PHF core and interfere with elongation by a still unknown mechanism. Whether compounds that block elongation are beneficial or not to the cell remains a matter of debate since it is currently unclear which species in the chain of aggregation causes toxicity. In the case of Aβ-aggregation the oligomeric species, rather than the fully developed fibers, are now considered the main toxic species [53], and certain experiments with Drosophila models of tauopathy are consistent with a similar interpretation [54].

A final point of discussion concerns the issue of phosphorylation. Alzheimer tau is characterized both by aggregation and by hyperphosphorylation involving numerous phosphorylation sites and kinases, and there is an ongoing debate on the relationship between the two aspects. The aggregation of tau in vitro does not require phosphorylation, and in fact phosphorylation is antagonistic to aggregation [14]. Thus most tau assembly studies have been performed with unphosphorylated protein. On the other hand, evidence from animal models suggests that tau phosphorylation could be toxic to neurons even without the formation of massive tangle pathology [54-56]. Thus future attention needs to be paid to the delicate balance between microtubule-bound tau, free tau, and the initial stages of aggregation. This may be regulated by phosphorylation [57], isof orm distribution (perturbed in FTDP-17, [58]), degradation by the proteasome or by caspases, [19, 59-61], or other mechanisms yet to be discovered.

ACKNOWLEDGEMENTS

We would like to thank Sabrina Hübschmann and Bianca Wichmann for excellent technical assistance. This project was supported in part by a grant from the Institute for the Study on Aging, New York.

ABBREVIATIONS

FRET = Fluorescence resonance energy transfer
AD = Alzheimer's disease
PHF = Paired helical filaments

REFERENCES