Aggregation Inhibitors

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Mechanistic Basis of Phenothiazine-Driven Inhibition of Tau Aggregation**

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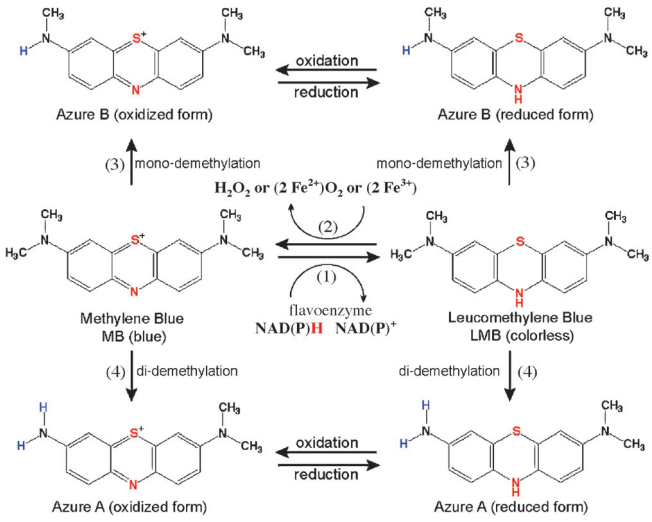
Alzheimer's disease (AD) is the most widespread dementia syndrome showing progressive presence of abundant deposits of extracellular senile β-amyloid polypeptide (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) consisting of Tau protein.[1,2] Tau protein is an intrinsically disordered protein that is abundant in neuronal axons where it promotes and stabilizes microtubule assembly.[3] With progression of AD, Tau aggregates and accumulates into NFTs.[2] As there is still no causative treatment or cure for AD and other tauopathies, Tau-based research aims to reveal the pathological consequences of amyloid formation and to implement new therapeutic strategies. In this effort, identification of inhibitors of tau aggregation as potential disease-modifying drugs and investigation of their mode of action play an important role.[4]

Methylene Blue (MB), a tricyclic phenothiazine also known as methylthionine hydrochloride,[5] has a history of diverse medical applications stretching back over 100 years, including use for distinctive cellular targets.[6] MB has been shown to prevent Tau aggregation in vitro[6–8] and to reduce the amount of Tau aggregates in a *C. elegans* model of Tau pathology.[9] This treatment has relieved Tau-induced toxicity of treated worms.[9] Moreover, MB progressed to phase 2 clinical trials in human AD patients with promising results[10] and was recently announced to enter a phase 3 clinical trials.[11] Despite these positive effects, care must be exercised because MB and its derivatives azure A and B have toxic side effects at elevated concentrations.[12] In model systems (e.g. Tau-expressing neuronal cells, *C. elegans* expressing Tau-V337M,[9] and zebra fish expressing Tau-P301S[13]) there are no detectable toxic effects at the concentrations used to inhibit Tau aggregation.

Besides inhibition of Tau aggregation other modes of action for MB in Alzheimer's disease were proposed, such as its antioxidant activity.[6] Moreover, MB inhibits oligomerization of amyloid-β peptide by promoting fibrillization and interferes with aggregation of the prion protein.[14,15] Mono- and di-*N*-demethylation of MB yield azure B and azure A, respectively (Scheme 1), which also have anti-aggregation and pharmacological effects.[8,12] Herein we reveal a distinct mechanism of action of MB and its metabolites azure A and azure B in the inhibition of Tau aggregation. We show that the mechanism of Tau aggregation inhibition is based on the interplay of reduction/oxidation of the native cysteine residues of Tau. MB and its metabolites prevent the formation of filaments and their toxic precursors by retaining Tau in a monomeric disordered conformation.

The IC_{50} values reported for inhibition of Tau-aggregation by MB varies from approximately 2 μM for four-repeat Tau to around 30 μM for three-repeat Tau.[6,8,16] To identify the residues of Tau that are essential for the interaction with MB, we used NMR spectroscopy. For increasing MB concentra-



Scheme 1. The reduction of MB yields leucoMB (1) and is regenerated in oxidizing conditions (2). Mono- and di-*N*-demethylation yield azure B (3) and azure A (4), respectively.

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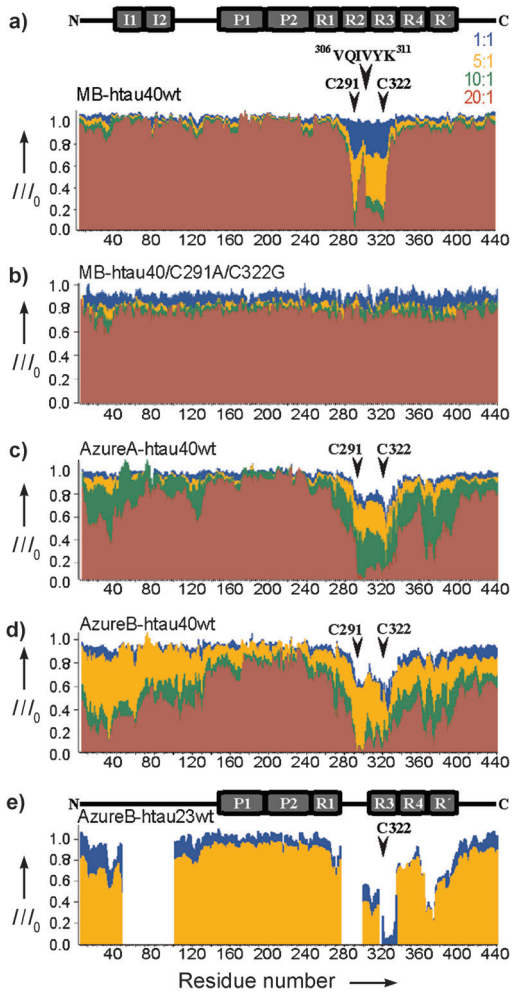

Figure 1. The Interaction of methylene blue with Tau. NMR intensity ratios I/I_0 (I = intensity of Tau resonances in presence of compound; I_0 = peak intensities of free Tau) from 2D ^1H–^{15}N HSQC spectra of htau40 (a–d). Signal broadening ($I/I_0 < 1.0$) is due to exchange of Tau between the free Tau conformations and those in the presence of the compound. (a) and (b) show the NMR broadening profile of wild-type and the cysteine-free variant of htau40 at increasing concentrations of MB (see color-coded panel for ligand:protein ratios). The same titration analysis with wild-type htau40 was conducted with azure A (c) and azure B (d). The domain organization of htau40 is shown above (I = insert, P = proline-rich region, and R = pseudo-repeat). e) NMR signal broadening in ^1H–^{15}N HSQC spectra of htau23 (lacking I1, I2, and R2) in the presence of azure B. All NMR experiments contained 100 μM protein and 1 mM DTT prepared in 50 mM phosphate buffer, pH 6.8.

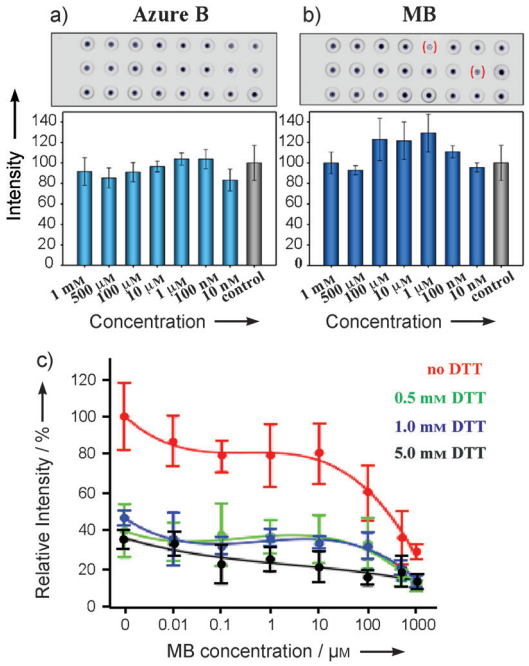

Figure 2. a, b) Filter assay and immunological detection of htau40/C291A/C322A (see also Figure S2) with azure B (a) and MB (b). Note, that azure B and MB interfere with ThS fluorescence precluding the use of ThS fluorescence for detection of filament formation. c) Aggregation of the three-repeat Tau construct K19 in the presence of different concentrations of DTT and MB measured by a filter assay (see also Figure S5). Details on sample preparations are available as Supporting Information. The intensity refers to the amount of aggregated Tau.

tions, changes in NMR signal position and intensity in two-dimensional ^1H–^{15}N heteronuclear single quantum coherence (HSQC) spectra of the 441-residue full-length Tau protein, htau40, were observed (Figure 1a and Figure S1 in the Supporting Information). Chemical shift changes were found in repeats R2 and R3 in the microtubule-binding domain and included the second hexapeptide ^{306}VQIVYK^{311} that is essential for aggregation of Tau. In addition, NMR signal perturbation was detected at the N-terminus in proximity to the aromatic residues Y18 and Y29. Most apparent, however, was the strong broadening of residues in proximity to the two native cysteine residues of htau40, C291 and C322 (Figure 1a). Substitution of the two cysteine residues by alanine and glycine, respectively, abolished the NMR line-broadening in the repeat region (Figure 1b), and MB lost its inhibitory effect on Tau aggregation (Figure 2a,b, Figure S2).

To obtain insight into the importance of the oxidation/reduction state of the cysteine residues of Tau, we added excess dithiothreitol (DTT) to the Tau–MB sample. Addition of DTT strongly decreased NMR signal broadening (see Figure S3), pointing towards MB-induced oxidation of the native cysteine residues of Tau. NMR spectroscopy and mass spectrometry demonstrated that MB modifies cysteine residues to sulfenic, sulfinic, and sulfonic acids (Figure 3a–c and Figure S4). In addition, MB and its derivatives contain a central thiazine ring and are thus able to undergo oxidation–reduction reactions.

Although at neutral pH values the auto-oxidation rate of the reduced form of MB, leucoMB, is very fast and leucoMB is not stable, dithiol compounds are able to produce leucoMB under quasiphysiological conditions (Figure S4).[17] As we used 1 mM DTT to keep the native cysteine residues of Tau in a reduced state, an increasing fraction of MB was in the oxidized state when the MB concentration was increased during the titration.

NMR spectroscopy revealed a diminished binding capacity of MB to Tau in the presence of DTT (Figure S3).

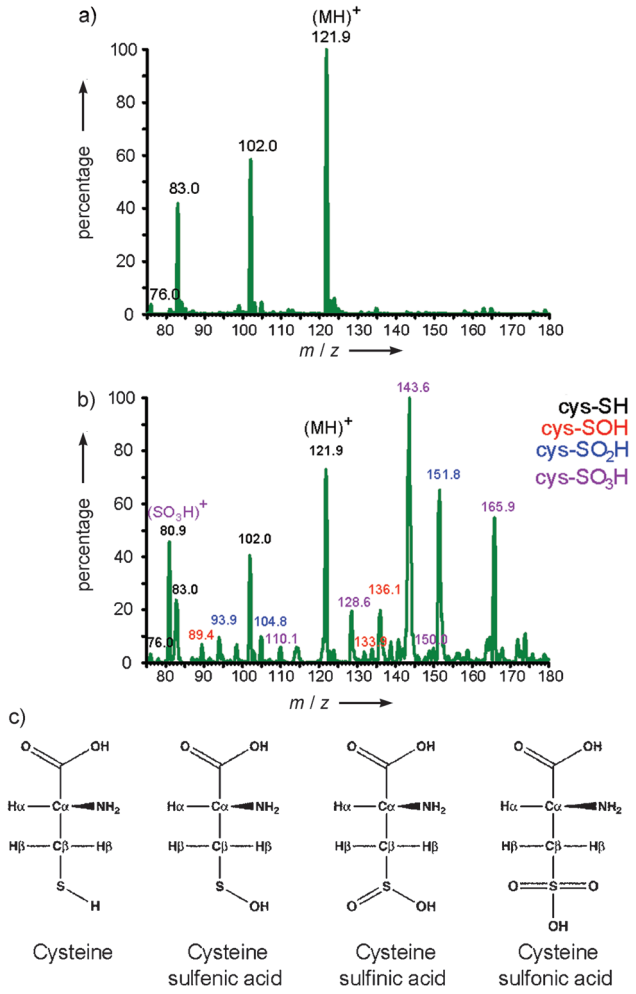

Figure 3. a, b) ESI-MS spectra of the free amino acid cysteine in the absence (a) and presence of MB (b). The color-coded fragments correspond to different oxidation states of the sulfhydryl group as represented by their structural formula shown in (c).

To answer the question if MB shows an inhibitory effect on Tau aggregation under reducing conditions, we performed a filter assay with the three-repeat Tau construct K19 (containing only one cysteine) in the presence of different DTT and MB concentrations. 10 μM of K19 protein treated with 2.5 μM $heparin_{3000}$ were incubated over 168 h with 0, 0.5, 1, and 5 mM DTT. The aggregation status of the MB-free sample was monitored by a Thioflavin S (ThS) assay (Figure S5). We observed diminished Tau aggregation in the presence of DTT. The ThS signal dropped to 36% of the DTT-free control. This is in line with a decrease of K19 dimer seeds by destruction of intermolecular disulfide bridges by DTT.

We tested the aggregation ability of K19 under different reducing conditions. MB was used in the range from 0 to 1 mM, aggregation was allowed to proceed for 168 h, and the aggregation status was monitored with a filter assay (Figure 2 c and S5). In the DTT-free control MB lowered the aggregation efficiency of K19 by about 70%. With increasing amounts of DTT the inhibition of Tau aggregation was diminished. At 0.5 mM and 1 mM DTT the value dropped to approximately 30%, and with 5 mM DTT to 25%. The experiments show that MB is able to inhibit the aggregation of three-repeat Tau under reducing conditions and in concentrations of DTT of up to 5 mM. In living cells the reducing function is provided by gluthatione (GSH), an antioxidant preventing damage to important cell components.

For the proper function in the cell cytosol, GSH often reaches low millimolar levels.[18] This situation suggests that the 5 mM concentration of DTT chosen in our in vitro experiment was comparable to a relatively high cellular GSH concentration. It is known that the concentration of GSH declines with age and age-related neurodegenerative diseases.[19] As a consequence the reducing potential in the cytosol will become weaker. Thus the lowering of the GSH concentration will favor the aggregation of Tau. Another factor favoring aggregation of Tau is oxidative stress that is elevated in neurons affected in neurodegenerative diseases and leads to a shift in the redox balance of GSH to less antioxidative values and thus lower antioxidative properties.[20]

As demethylation of MB is favored by several in vitro and in vivo conditions,[5] we probed the interaction of the didemethylated and monodemethylated derivatives of MB, azure A, and azure B (Scheme 1), with htau40. At low concentrations, azure A and B led to NMR signal broadening next to the two htau40 cysteine residues (Figure 1 c,d) which corresponds to their oxidation. However, at higher compound concentrations and in striking contrast to MB, azure A and azure B also showed strong signal broadening in regions containing aromatic residues, such as the N-terminal tail and residues 360–400 (^{16}GTYG^{19}, ^{27}GGYT^{30}, ^{114}LEDEAAGHVT^{123}, ^{362}HVPGGG^{367}, ^{371}IETH^{374}, and ^{386}TDHGAEIVYKS^{396}). A similar effect was observed for the three-repeat isoform of Tau, htau23, that contains only the single cysteine C322 and for which strong signal broadening was observed for C322 as well as ^{362}HVPGGG^{367} and ^{371}IETH^{374} (Figure 1 e). The reason for binding of azure A and azure B and not MB to the aromatic residues of Tau, lies in the hydrophilic nature of the MB molecule, which has been suggested to be responsible for the finding that MB is not an efficient stain for normal blood cells in contrast to azure B.[5] Thus, upon demethylation of MB new modes of interaction and aggregation inhibition are possible in line with the finding that azure A and B are 30-fold more potent than MB in inhibition of Tau aggregation.[7]

The ^{1}H–^{15}N HSQC spectrum of htau40 in the presence of MB, azure A and azure B retained its low dispersion and chemical shifts characteristic of a disordered protein. To obtain further insight into the Tau species that are stimulated by MB we performed circular dichroism (CD) and small angle X-ray scattering (SAXS) measurements. CD spectroscopy demonstrated that azure B did not induce detectable amounts of regular secondary structure in Tau (Figure 4 a). SAXS showed that even at large excess of azure B, Tau is a highly disordered protein (Figure 4 b,c). The SAXS-derived radius of gyration, R_g of (6.7 ± 0.3) nm (at fivefold excess of azure B), was comparable to that of monomeric Tau,[21] demonstrating that the MB–Tau interaction does not result in the formation of Tau aggregates. Indeed, determination of the hydrody-

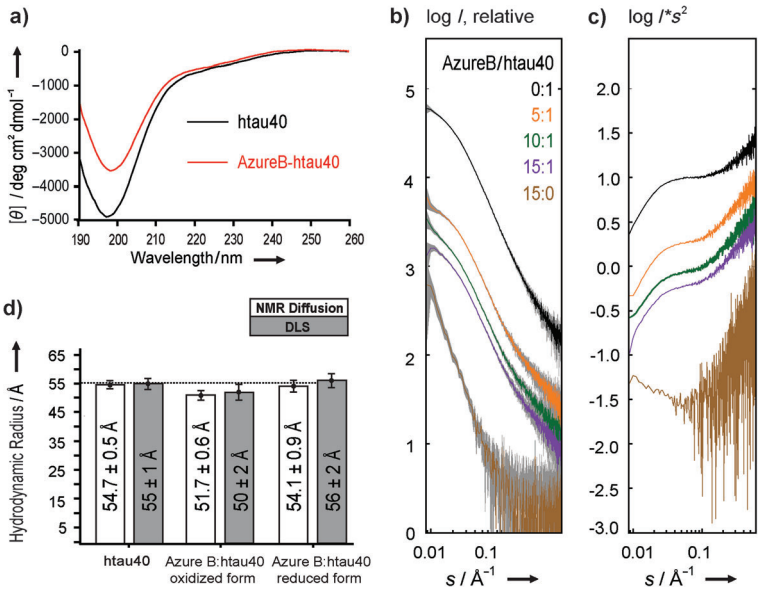

Figure 4. MB and its metabolites retain Tau in a monomeric disordered state. a) Far UV CD spectra of htau40 in the absence (black) and presence of azure B (20:1, red). b) SAXS profiles and c) Kratky plots of htau40 alone (black) and together with azure B at increasing ligand:protein ratios (color-coded panel). $s = 4\pi \sin(\theta)/\lambda$, where θ is half the scattering angle and $\lambda = 1.5$ Å is the X-ray wavelength on X33. Curves are off-set arbitrarily for clarity (with normalized values on the *y*-axis). d) Hydrodynamic radius of htau40 in the absence and presence of a 20-fold excess of azure B as determined by NMR spectroscopy and DLS, and after addition of 8 mM DTT (right). Details on sample preparation are available as Supporting information.

namic radius by pulsed-field gradient NMR spectroscopy[22] and dynamic light scattering (DLS) pointed to a slight compacting of the ensemble of Tau conformers that was released in the presence of excess DTT (Figure 4d). We conclude that MB and its derivatives azure A and B retain Tau in a monomeric aggregation incompetent state.

An important aspect of aggregation inhibition is that it can potentially interfere with the physiological function of the protein. As Tau is a microtubule-associated protein,[23] interference of phenothiazines with the ability of Tau to promote microtubule assembly might be detrimental in cells. However, azure A does not interfere with the Tau–tubulin interaction when introduced at a 1000-fold excess with respect to Tau,[7] and MB did not influence Tau-promoted microtubule assembly.[8,24] Thus, the aggregation incompetent monomeric conformation of Tau induced by MB and its derivatives is able to efficiently interact with microtubules.

Our data demonstrate that MB and its N-demethylated derivatives azure A and azure B modify the two native cysteine residues of Tau to sulfenic, sulfinic, and sulfonic acid. The modification of protein cysteine residues through reversible oxidation of cysteine sulfhydryl groups and the formation of sulfenic acids are crucial regulatory events in biological systems.[25] The nucleophilic character of the sulfhydryl group results in several distinct redox pathways and facilitates roles in electron donation, hydride-transfer reactions, and free radical reactions. Along these pathways, disulfide bridge formation is the most dominant chemical oxidation as it enhances structural stability. In case of Tau it was shown that intramolecular cross-linking of C291 and C322 in four-repeat isoforms of Tau (such as htau40) strongly inhibits Tau aggregation, whereas formation of intermolecular disulfide bonds in three-repeat isoforms of Tau, for example, htau23, promotes aggregation.[26] In striking contrast, we demonstrate that modification of the cysteine residues to sulfenic, sulfinic, and sulfonic acid converts both four-repeat and three-repeat Tau to an aggregation incompetent, monomeric state (Figure 3, 4): MB inhibits aggregation of both four-repeat Tau and three-repeat Tau with IC_{50} values of approximately 2 μM[8,16] and 30 μM,[6] respectively. The lower IC_{50} value for three-repeat Tau is in line with the presence of a single cysteine residue, C322, that is, a single modification site, in comparison to the two cysteine residues of htau40. The specific modification of the cysteine residues is critical for aggregation inhibition as substitution of the cysteine residues by alanine did not interfere with filament formation (Figure S2), whereas it abolished the inhibitory effect of MB on Tau aggregation (Figure 2a,b).

In summary we have provided mechanistic insights into the inhibition of Tau aggregation by phenothiazines. Specific modification of the native cysteine residues retains Tau in a monomeric conformation preventing the formation of filaments and their toxic precursors. Demethylation of methylene blue establishes new interactions with Tau and enables additional means for modulation of Tau aggregation.

Experimental Section

Unlabeled and labeled wild-type and mutated human Tau proteins were expressed and purified as described in the literature.[27] MB, azure A and B were purchased from MP Biomedicals (MP Biomedicals S.A. Heidelberg, Germany). NMR experiments were recorded at 5 °C on Bruker Avance 700 MHz or Avance III 800 MHz spectrometers. NMR samples contained ^{15}N-labeled Tau(100 μM) in phosphate buffer (50 mM; pH 6.8), DTT (1 mM), and 10 % (v/v) D_2O. SAXS data were collected at X33 at the European Molecular Biology Laboratory on DORIS III (DESY) at a wavelength of 1.5 Å at 25 °C using a Pilatus 1M photon counting detector. Samples were prepared in phosphate buffer (50 mM) and contained htau40 (100 μM; 4.5 mg mL^{-1}) and DTT (1 mM). Full details are available in the Supporting Information.

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