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Potency of a tau fibrillization inhibitor is influenced by its aggregation state

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Abstract

Tau fibrillization is a potential therapeutic target for Alzheimer's and other neurodegenerative diseases. Several small molecule inhibitors of tau aggregation have been developed for this purpose. One of them, 3,3'-bis(β-hydroxyethyl)-9-ethyl-5,5'-dimethoxythiacarbocyanine iodide (N744), is a cationic thiacarbocyanine dye that inhibits recombinant tau filament formation when present at submicromolar concentrations. To prepare dosing regimens for testing N744 activity in biological models, its full concentration-effect relationship in the range 0.01 – 60 μM was examined *in vitro* by electron microscopy and laser light scattering methods. Results revealed that N744 concentration dependence was biphasic, with fibrillization inhibitory activity appearing at submicromolar concentration, but with relief of inhibition and increases in fibrillization apparent above 10 μM. Therefore, fibrillization was inhibited ≥50% only over a narrow concentration range, which was further reduced by filament stabilizing modifications such as tau pseudophosphorylation. N744 inhibitory activity also was paralleled by changes in its aggregation state, with dimer predominating at inhibitory concentrations and large dye aggregates appearing at high concentrations. Ligand dimerization was promoted by the presence of tau protein, which lowered the equilibrium dissociation constant for dimerization more than an order of magnitude relative to controls. The results suggest that ligand aggregation may play an important role in both inhibitory and disinhibitory phases of the concentration-effect curve, and may lead to complex dose response relationships in model systems.

Keywords

Alzheimer's disease; tau; neurofibrillary tangle; cyanine dye; aggregation

Introduction

Protein misfolding and self-association accompanies several neurodegenerative diseases, including Alzheimer's and Huntington's diseases [1]. The filamentous aggregates appearing in both of these disorders correlate both spatially and temporally with disease signs, suggesting that protein aggregation is closely associated with cellular misfunction and cytotoxicity [2]. For this reason, small-molecule inhibitors of protein aggregation are under investigation as potential therapeutic agents. These include azo dyes such as Congo red [3–6], phenothiazines,

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and other aromatic heterocycles [4,7]. The interaction of these compounds with self-aggregating proteins is complex. Each can associate with mature filaments through high-affinity binding sites distributed along the filament long axis [8,9]. However, some also bind partially folded conformations intermediate between protein monomer and filament [10–12] and to oligomeric species potentially off the fibrillization pathway [13,14]. As a result, compounds such as Congo red appear to induce or inhibit protein aggregation, depending on the target investigated [4,11,15,16]. Furthermore, within a single protein target, induction and inhibition can predominate in different concentration regimes, potentially leading to intricate dose response relationships *in vitro* and in biological models. For example, in brain slice preparations derived from a mouse model of Huntington's disease, Congo red activity is triphasic [17]. At submicromolar concentrations, the dye partially antagonizes fibrillization of aggregation-prone huntingtin fragments. As concentrations rise, however, inhibition is lost, and Congo red actually increases huntingtin aggregation. Finally, in the high concentration regime, neither inhibition nor aggregation inducing activity is apparent [17]. These results illustrate the importance of dosing regimens for testing and interpreting the pharmacology of such ligands in biological models, and the need to assess the mechanisms through which these effects occur.

Recently, we investigated the interaction of aromatic heterocycles with tau protein [18,19], the principal component of neuritic lesions that develop within vulnerable neurons in Alzheimer's disease [20]. Tau is a natively unfolded monomer that aggregates to form filaments within which a portion of each molecule adopts β -sheet conformation. Like other filamentous aggregates, the resultant β -sheets stack in parallel, with each sheet orthogonal to the axis of the growing tau filament [21]. Under near physiological conditions *in vitro*, full-length tau protein requires the presence of exogenous inducing agents to adopt this conformation and aggregation state over experimentally tractable time periods [22]. These agents, which include anionic surfactant micelles and anionic microspheres, promote heterogeneous nucleation (i.e., nucleation owing to the presence of foreign particles) on their surfaces from which tau filaments then extend in an elongation reaction [15,23,24]. In the low concentration regime, neither Congo red nor benzothiazole derivatives such as thioflavin S modulate poly-anion induced aggregation of full-length monomeric tau proteins [15]. In the high concentration regime, however, these dyes become efficacious fibrillization inducers, perhaps because of their ability to bind and organize β -sheet structure [11], and can completely replace the need for poly-anions [15]. In contrast, cyanine dyes such as N744 inhibit polyanion-induced tau aggregation at substoichiometric concentrations [18,19]. We have postulated that dye aggregation contributes to the differential activity of certain aromatic heterocycles on tau filament formation. Aggregation is common in many structural classes of dyes, and is influenced by pH, ionic strength, temperature, and solvent polarity, as well as dye structure and concentration [25]. Like tau protein, dye aggregation proceeds through a nucleation dependent mechanism [26]. Alone in solution, primary nucleation is homogeneous: dye molecules self associate directly without the participation of other molecules. Cyanine aggregation is influenced, however, by the presence of substrates including silver halide salts [27], duplex DNA [28], and proteins containing extended β -sheet structure [29,30]. Cyanine spectral properties vary with each of these substrates, suggesting that nucleation conditions can influence cyanine molecular organization.

Here we investigate the concentration dependence of cyanine dye N744-mediated modulation of polyanion-induced tau aggregation under near physiological conditions *in vitro*. The results recapitulate the complex dose response relationship observed previously for Congo red acting on huntingtin in a biological model, and suggest the mechanistic basis of the effect in the context of cyanine dyes.

Materials and Methods

Materials

Recombinant His-tagged wild-type htau40 (*i.e.*, the longest human central nervous system isoform of tau, [31]) and pseudophosphorylation mutant htau40^{T212E} were prepared as described previously [32,33]. Mixed histones (type II-A from calf thymus) were from Sigma (St. Louis, MO). Stock solutions of alkyl sulfate detergent inducer C₁₈H₃₇SO₄Na (Research Plus, Bayonne, NJ) were prepared in 1:1 H₂O/isopropanol. Glutaraldehyde, uranyl acetate, and 300 mesh carbon-coated copper grids were from Electron Microscopy Sciences (Ft. Washington, PA). Stock solutions of N744 (custom synthesized by deCODE Genetics, Lemont, IL) were prepared in DMSO. Carboxylate-conjugated polystyrene microspheres (90 nm diameter, molecular area = 12 Å²/eq) were from Bangs Laboratories, Inc (Fishers, IN).

Aggregation Assays

Under standard conditions, protein preparations were incubated without agitation in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol) in the presence or absence of fibrillization inducers (C₁₈H₃₇SO₄Na, or carboxylate-modified microspheres) and inhibitor N744. Control reactions were normalized for DMSO vehicle, which was limited to 2% (v/v) final concentration in all aggregation reactions.

Reactions were performed at either 37°C (for analysis by transmission electron microscopy) or 22°C (for laser light scattering). N744 potency is similar at these temperatures [18,19]. For analysis by transmission electron microscopy, aliquots were removed, treated with 2% glutaraldehyde (final concentration), mounted on formvar/carbon-coated 300 mesh grids, and negatively stained with 2% uranyl acetate as described previously [24,34]. Random images were viewed in a Phillips CM 12 transmission electron microscope operated at 65 kV, captured on film at 8,000 – 60,000-fold magnification, digitized, and imported into Optimas 6.5.1 for quantification of filament lengths and numbers [34]. Individual filaments were counted manually. Reaction products were analyzed by laser light scattering as described previously [35].

Dye Aggregation

N744 (0.2 – 30 μM total concentration) was incubated (1 h at 37°C) in assembly buffer containing either 4 μM htau40 or 0.6 μM mixed histones, then subjected to absorbance spectroscopy (Varian Cary 50 Bio) over the wavelength range 450 – 700 nm. Concentrations of dye monomer (C_m) were estimated as described previously [19,36]. Briefly, the extinction coefficient for N744 monomer was established in methanol, then used to estimate monomer concentration in aqueous solutions after deconvolution of absorbance data into overlapping Gaussian peaks using Beer-Lambert law:

$$A = \epsilon C_m l \quad (1)$$

Where A is absorbance, ϵ is the molar extinction coefficient, and l is the path length of the sample. N744 dimer concentration (C_d) was estimated from total dye concentration (C_t) and the assumption [36]:

$$C_d = (C_t - C_m) / 2 \quad (2)$$

The dissociation constant for dimerization (K_{dim}) was then calculated from the function [36]:

$$K_{dim} = C_m^2 / C_d \quad (3)$$

K_{dim} values were derived from at least three spectra and reported as mean $\pm$ S.D.

Analytical Methods

The volume occupied by tau adsorbed to anionic microsphere surfaces (V_{shell}) was approximated from negatively stained transmission electron microscopy images and the function:

$$V_{\text{shell}} = 4/3\pi(r^3 - r_0^3) \quad (4)$$

where r_0 is the radius of microspheres determined in the absence of tau protein and r is the radius of microspheres decorated with tau protein. Values are reported $\pm$ S.D. of replicate measurements.

Results

N744 inhibitory activity is biphasic

N744 is a thiacyanine dye (Fig. 1) that inhibits polyanion-induced aggregation of full-length tau isoform htau40 at substoichiometric concentrations [18,19]. To examine its effects on tau aggregation over a broad concentration range, the concentration dependence was determined in the presence of anionic surfactant inducer $\text{C}_{18}\text{H}_{37}\text{SO}_4\text{Na}$ under standard, near-physiological conditions of pH, ionic strength, bulk tau concentration (4 μM) and redox environment. $\text{C}_{18}\text{H}_{37}\text{SO}_4\text{Na}$ was chosen as inducer because it permits analysis of aggregation by both electron microscopy and laser light scattering methods [35,37]. Results showed that N744 potently inhibited tau aggregation with an IC_{50} of ~ 300 nM under these conditions (Fig. 2), consistent with previously reported values [18,19]. As N744 concentrations rose above 10 μM , however, inhibition was relieved, with the IC_{50} retraced at ~ 13 μM . At still higher concentrations, tau aggregation was enhanced to levels above reactions performed in the complete absence of inhibitor (Fig. 2). These data suggest that the concentration-effect curve for N744 is complex, with both inhibitory and stimulatory phases in distinct concentration regimes. As a result, there is a limited concentration range over which N744 is active as an inhibitor, with the ratio of IC_{50} values found in each phase being ~ 43 for wild-type htau40.

To confirm these findings, the influence of N744 on htau40 aggregation was examined in the presence of anionic microsphere inducer. Unlike anionic surfactants, microspheres do not undergo micellization or otherwise change structure during the aggregation reaction [23], thereby eliminating these variables from the analysis. Electron micrographs showed that N744 drove a concentration-dependent depression of total filament length when present at concentrations up to 3 μM (Fig. 3). Inhibition was relieved above 10 μM , however, with large numbers of short filaments becoming apparent (Fig. 3). These data confirm the biphasic nature of N744 activity, and demonstrate that the effect is not unique to or dependent on micellar inducers.

Mechanism

In the low concentration regime, N744 inhibits tau fibrillization by raising the apparent concentration of tau required to support the aggregation reaction (*i.e.*, the “critical concentration”, Ref. [19]). This may occur directly through binding of reacting species or indirectly through stabilization of off-pathway species as proposed for inhibitors of α -synuclein [13]. The biphasic concentration effect relationship shown in Fig. 2 predicted that N744 effects on critical concentration would reverse in the high N744 concentration regime. To test this prediction, critical concentration was estimated from the dependence of fibrillization on bulk tau concentration [38] in the presence of up to 20 μM N744. Results showed that critical

concentration increased with N744 up to an optimum of $\sim 4 \mu\text{M}$, then reversed and lowered as N744 concentration continued to rise (Fig. 4). These data suggest that the ability of N744 to modulate critical concentration was consistent with its biphasic effects on tau filament formation.

Why did N744 effects on critical concentration reverse above $\sim 4 \mu\text{M}$? One possibility was that N744 became a tau aggregation inducer in the high concentration regime much like Congo red and thioflavin S [15]. To test this hypothesis, httau40 was incubated under standard conditions with varying concentrations of N744 in the absence of anionic inducer. On the basis of electron microscope analysis, N744 did not induce aggregation at any tested concentration up to $100 \mu\text{M}$ (data not shown). These data suggest that the increase in total filament mass seen at high concentrations of N744 did not result from direct stimulation of aggregation.

A second possibility was that dye activity decreased owing to its sequestration into higher order complexes. Thiocarbocyanine derivatives self associate in both aqueous solution and on solid surfaces owing to strong dispersion forces between their nearly planar faces [27]. Shifts in absorbance spectra accompany thiocarbocyanine aggregation depending on the quaternary structure of the aggregate formed. Hypsochromic shifts to shorter wavelengths are generally referred to as H-bands. In solution, these consist of small aggregates (dimer, trimer, etc) but much larger sizes can be attained especially in the presence of substrates [39]. Bathochromic shifts to longer wavelengths are termed J-bands. Although both classes of aggregate are composed of parallel dye molecules stacked plane-to-plane, they differ in the angle of slippage between successive molecular planes [19].

To determine the aggregation state of N744, its absorbance spectrum was measured as a function of concentration under various conditions. In neat methanol, which does not support dye oligomerization [36] and therefore calibrates monomer concentration, N744 absorbance appeared as a major band at 568 nm with a weak vibrational shoulder at 547 nm (Fig. 5A). N744 absorbance patterns were then measured in aqueous solution under aggregation promoting conditions. Because anionic surfactant micelles alone influence the aggregation state of thiocarbocyanine dyes [40–42], N744 absorbance was first analyzed in reactions where tau was replaced with mixed histones, a protein preparation that induces surfactant micellization but that does not fibrillize under standard assay conditions [35]. Histone concentrations were chosen on the basis of Corrin-Harkins plots [23] to yield the same surfactant critical micelle concentration as that resulting from the presence of tau protein [35]. Thus, the histone-containing reaction served to control for non-specific, micelle-associated changes in N744 absorbance. Results showed that at low bulk N744 concentrations of $200 - 600 \text{ nM}$, absorbance spectra were dominated by the presence of N744 monomer centered at $585 \pm 2 \text{ nm}$ (mean $\pm$ S.D. of three spectra; Fig. 5A). The bathochromic shift in monomer absorbance in the presence of alkyl sulfate detergent micelles relative to methanol is consistent with established behavior of cationic thiocarbocyanine dyes [43]. Nonetheless, as bulk N744 concentrations increased, the relative intensity of the monomer band steadily decreased while the relative intensity of H-aggregates increased (Fig. 5A). To characterize these aggregates, the amount of monomer as a function of bulk N744 concentration was determined by absorbance spectroscopy and used to calculate the concentration of dye protomer in aggregate form. A double log plot of monomer *versus* protomer concentration was linear with a slope of 2.4 ± 0.4 , indicating that the predominant aggregate formed in this concentration range was a dimer (Figure 6). On the basis of equations 2 and 3 (see Materials and Methods), the dissociation equilibrium constant for dimerization (K_{dim}) was $2.2 \pm 0.5 \mu\text{M}$. These data demonstrate that the N744 dimerization constant in the presence of micelles is similar to the value estimated in assembly buffer alone ($2.1 \pm 0.4 \mu\text{M}$; [19]). As N744 concentrations rose above 600 nM , however, broad H-bands appeared at shorter wavelengths (Fig. 5A), and the slope of double log monomer versus protomer plots steepened markedly

(Fig. 6). These data are consistent with higher order aggregates forming as bulk N744 concentrations increased. Together, these data indicate that the aggregation behavior of N744 in the presence of $C_{18}H_{37}SO_4Na$ micelles conforms to the pattern established for the interaction of cationic thiacyanine dyes with alkyl sulfate micelles [40].

The presence of tau protein modified this pattern. At 200 nM bulk N744, levels of dye monomer, which appeared at 592 ± 1 nm (mean $\pm$ S.D. of three spectra; Fig. 5B), were well below those observed in the presence of histone, suggesting that N744 underwent significant aggregation under these conditions. A double log plot of monomer *versus* protomer concentration was linear with a slope of 2.0 ± 0.1 (Fig. 6), indicating that the predominant aggregate formed in the concentration range of 200 – 800 nM bulk N744 was dimer. Consistent with this, the spectrum revealed the presence of a major dimer band at ~ 540 nm (Fig. 5B). On the basis of equations 2 and 3, K_{dim} was estimated as 0.15 ± 0.01 μM . These data indicate that the presence of tau/ $C_{18}H_{37}SO_4Na$ supported N744 dimerization at submicromolar concentrations, a full order of magnitude lower than in assembly buffer alone, assembly buffer containing htau40, or $C_{18}H_{37}SO_4Na$ micelles stabilized by histone [19]. As bulk N744 concentrations rose above 1 μM , however, a third major absorbance peak centered on ~ 513 nm became apparent as monomer and dimer peaks decreased. These data indicate that higher order aggregates form as bulk N744 concentrations rise above 1 μM at the expense of monomeric and dimeric species. By 30 μM bulk N744 concentration, the absorbance pattern was dominated by H-aggregate formation whereas monomer and dimer peaks became indistinct. These data suggest that the loss of fibrillization-inhibition observed above 10 μM N744 may result from the sequestration of N744 into large supramolecular complexes.

Although dye sequestration may account for loss of inhibitory activity at high bulk N744 concentrations, it does not explain why the amount of fibrillar material overshoot levels of fibrillization measured in the complete absence of inhibitor. The system behaved as if more tau were available for aggregation. On the basis of transmission electron microscopy images of microsphere-mediated tau aggregation, tau distributes into at least three pools. Bulk tau initially consists of soluble protein, but with time appears to coat the surface of microspheres and also to form filaments extending from their surfaces [23]. Because N744 does not directly induce filament formation from the soluble pool, the increase in the filamentous pool is likely derived from the microsphere bound pool. To test this hypothesis, the amount of tau coating individual microspheres was estimated in the presence of varying concentrations of N744 by transmission electron microscopy. In the absence of N744, microspheres appeared as roughly spherical objects of diameter 55 ± 2 nm (Fig. 7A). The presence of N744 up to 30 μM did not change these values (data not shown), which appeared to differ from manufacturer's specifications made on the basis of hydrodynamic analysis under different solvent conditions. In the presence of tau protein, however, beads became coated with negatively staining material, and appeared larger than beads photographed in the absence of tau (Fig. 7B). The calculated net volume difference between the two microsphere populations (i.e., the shell volume, V_{shell}) was then examined as a function of N744 concentration (Fig. 7C). Results showed that as N744 rose above 1 μM bulk concentration, the amount of negatively staining material coating the microspheres gradually decreased. These data indicate that the population of tau coating the microspheres decreases as bulk N744 concentrations rise. They further suggest that the large cationic N744 aggregates formed at high bulk N744 concentrations were capable of competing with tau for binding to the microsphere surface, thereby making more tau available for filament formation.

Effect of tau post-translational modification

AD-derived tau differs from normal tau in being extensively phosphorylated [44]. The site-specific incorporation of negative charge that accompanies phosphorylation promotes tau

aggregation by releasing tau from microtubules [45–47] while increasing the driving force for filament nucleation and extension by decreasing critical concentration (K_{crit} ; [32]). The latter effect can lower the effective potency of aggregation inhibitors such as N744 [19]. To mimic the effect of tau phosphorylation on the biphasic activity of N744, the aggregation of pseudophosphorylation mutant htau40^{T212E} was examined in the presence of $C_{18}H_{37}SO_4Na$ inducer. Htau40T212E was chosen for analysis because it returns the largest change in critical concentration of any phosphorylation mimicry mutant studied to date, thereby assuring that any differences in N744 potency would be large enough to measure [32]. Moreover, it binds N744 with similar affinity as htau40 [48], eliminating differences in binding affinity as a variable. Under standard conditions, the IC_{50} for N744 inhibition of htau40^{T212E} fibrillization was ~600 nM (Fig. 2), confirming that N744 potency is sensitive to modifications that modulate equilibria at filament ends [19]. As N744 concentrations rose, inhibition was relieved, with the IC_{50} retraced at ~10 μ M bulk N744 (Fig. 2). Compared to wild-type htau40, therefore, IC_{50} retracement for htau40^{T212E} occurred at a lower bulk N744 concentration. Overall, the ratio of IC_{50} values found for each phase was ~17, less than half the ratio found for wild-type htau40. These data indicate that the aggregation promoting effects of pseudophosphorylation are apparent in both phases of N744 action, and that post-translational modifications can significantly narrow the effective concentration range of compounds such as N744.

Discussion

The results presented here suggest that ordered aggregate formation can greatly influence the ability of cyanine dyes to modulate tau fibrillization. They also confirm that certain exogenous macromolecules can greatly affect the extent and nature of dye aggregation at submicromolar concentrations. Together these data implicate dye aggregation as a potential source of complexity for pharmacological modulation of protein fibrillization reactions. In fact, aggregation may be important for both the inhibitory and disinhibitory phases of cyanine action. With respect to the inhibitory phase, abnormally steep concentration-effect curves have been observed for many inhibitory ligands in protein fibrillization studies [4,7,49]. These may result from high protein concentrations relative to ligand binding affinity, which drive the reaction into Goldstein's Zone C, where concentration effects appear steep because inhibition is directly proportional to ligand concentration [50]. Protein aggregation reactions are complex, however, and involve multiple potential binding partners including partially folded intermediates [51]. It is conceivable that the binding target is present at low concentration and that steep concentration-effect relationships reflect a form of cooperativity. We have argued that the modestly steep concentration-effect curve for N744-mediated inhibition of tau fibrillization reflects cooperativity owing to ligand dimerization, in part because of the time-dependent appearance of dimer in the presence of tau protein and surfactant fibrillization inducer [19]. Indeed, steep concentration effect relationships are frequently observed when ligands aggregate as part of their mechanism of action [52]. Here we examined the concentration dependence of the effect, and found that tau in the presence of anionic micelles (i.e., conditions that generate assembly-competent tau conformations) greatly stabilized N744 dimer formation. In fact, K_{dim} decreased >10-fold relative to K_{dim} determined in the presence of $C_{18}H_{37}SO_4Na$ micelles or tau in the presence of assembly buffer alone. Most importantly, K_{dim} decreased below the IC_{50} for inhibition of tau fibrillization. Because anionic micelles function to stabilize assembly-competent tau conformation enriched in β -sheet content [15], and some cyanine dyes selectively interact with β -sheet structure [29,53], it is conceivable that this structural motif on assembly-competent tau serves as the template for N744 dimerization. In the presence of tau template, very low bulk N744 concentrations supported dimerization, with histone unable to substitute for tau in this respect. Dimers may be responsible for inhibitory activity since their concentration rises non-linearly with increasing bulk cyanine concentration in the submicromolar concentration regime.

As concentrations rose above 10 μM , however, aggregation abruptly shifted to large H-aggregate formation. This was accompanied by decreases in the relative concentration of monomers and dimers and also by losses in tau aggregation inhibitory activity. At the highest N744 concentration tested (30 μM), dimer was barely detectable, suggesting that it was consumed in the formation of large H-aggregates under these conditions. Large aggregate formation may therefore be responsible for loss of inhibitor activity in the high concentration regime through sequestration of inhibitory species. The presence of anionic surfactant micelles and tau protein may be responsible for the complex N744 aggregation behavior observed at different concentrations.

These data are important for design and interpretation of inhibitor trials in biological systems. Neurofibrillary lesion densities correlate with cognitive decline [54], and tau aggregation may be directly toxic to neurons that develop them. Studies in transgenic systems have been complicated, however, by the discovery that mere overexpression of normal tau can lead to neurodegeneration [55]. Because most biological models of tauopathy rely on overexpression of normal or mutant tau proteins to generate lesions or a neurodegenerative phenotype [56, 57], the source of toxicity observed in these systems can be ambiguous. Resolving this issue is crucial because tau expression is not upregulated in authentic AD tissue [58]. Selective fibrillization inhibitors may help clarify issues of toxicity. Results found here suggest that appropriate dosing in this effort will be critical for assessment of compounds such as N744. Both supraphysiological protein concentrations and post-translational modifications such as phosphorylation [32] and C-terminal truncation [59] will antagonize the critical-concentration raising activity of cyanines and reduce their potency. At the same time, higher order N744 aggregate formation may limit therapeutic index to a rather narrow window of effective concentrations. As a result, fibrillization antagonists may provide only partial antagonism under optimal dosing conditions. Similar considerations may rationalize the behavior of Congo red in models of Huntington's disease. *In vitro*, Congo red inhibition of huntingtin aggregation is monophasic with a steep concentration-effect relationship [4]. In biological models, however, complex dose response relationships similar to those found here were observed [17]. While it has been shown that Congo red and other aromatic heterocycles capable of interacting with proteins in cross- β -sheet conformation form disordered micelles at millimolar concentrations in aqueous solution [60,61], these compounds also form ordered aggregates at far lower, pharmacologically relevant concentrations. For example, the K_{dim} for Congo red has been estimated as $\sim 20 \mu\text{M}$ in water [62]. The results presented here suggest, however, that K_{dim} can vary with the presence of macromolecules. Large H-aggregate formation also depends on environment, as none are observed at 5 μM N744 in assembly buffer alone [19] whereas the presence of negatively charged surfaces such as anionic micelles drive extensive dye aggregation at this concentration (herein and [40]). Thus, even compounds that behave well *in vitro* can have complex dose response relationships in biological systems owing to heterogeneous nucleation of various aggregates. Dissociating aggregation propensity from inhibitory activity will simplify drug trials in biological model systems.

Finally, these data raise a caveat for interpretation of future structural studies involving ligands such as N744. Both X-ray crystallography and nuclear magnetic resonance studies require high protein and ligand concentrations for analysis. The structures obtained under these conditions may reveal aggregated rather than inhibitory conformations of ligand.

In summary, cyanine dye N744 displays complex concentration-effect behavior *in vitro*, and is an effective tau fibrillization inhibitor only over a limited concentration range. The behavior appears related to dye aggregation, which may be important for both inhibitory and disinhibitory concentration ranges, and suggests the basis for complex dose response data generated in biological systems for some protein aggregation inhibitors.

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Abbreviations used

AD

Alzheimer's disease

C₁₈H₃₇SO₄Na

sodium octadecyl sulfate

DTT

dithiothreitol

N744

3,3'-bis(β-hydroxyethyl)-9-ethyl-5,5'-dimethoxythiacarbocyanine iodide

Figure 1. N744 structure
N744 is a cationic thiocarbocyanine dye.

Figure 2. N744 action displays a biphasic concentration dependence

Wild-type tau40 (●) and pseudophosphorylation mutant tau40^{T212E} (○) were each incubated (4 h at 22°C) without agitation at 4 μM concentration in assembly buffer containing C₁₈H₃₇SO₄Na inducer (50 μM) and various concentrations of N744. Aliquots were then removed, stained with uranyl acetate, and viewed by electron microscopy as described in Materials and Methods. Each point represents total length of all filaments expressed as a normalized percentage of filament lengths measured in the presence of DMSO vehicle alone (triplicate determination ± S.D.), whereas the solid lines are drawn solely to aid visualization. Under these conditions, the effects of N744 on fibrillization of both tau40 and tau40^{T212E} followed a biphasic concentration dependence.

Figure 3. N744 action in the presence of anionic microsphere inducer

Wild-type htau40 (4 μM) was incubated (24 h at 37°C) in assembly buffer containing carboxylate-substituted polystyrene microspheres (124 pM) and varying concentrations of N744 (0, 1, 3, 10, or 30 μM as labeled in upper left corner of each panel). Aliquots were then removed and visualized by electron microscopy. In the absence of N744, tau formed filaments extending from the microsphere surface. Increasing concentrations of N744 initially decreased tau filament formation, but further increases relieved inhibition. These data suggest that the biphasic nature of N744 concentration dependence is not unique to surfactant inducers. Bar = 500 nm.

Figure 4. N744 modulates fibrillization critical concentration

The critical concentration (K_{crit}) of wild-type htau40 fibrillization was estimated as a function of bulk N744 concentration (0, 1, 4, 7, 20 μM) by static laser light scattering spectroscopy as described in Materials and Methods. The ability of N744 to modulate critical concentration displayed a biphasic concentration dependence, with maximal activity apparent at ~4 μM N744.

Figure 5. N744 absorbance spectra

Varying concentrations of N744 (0.2 – 30 μ M) were incubated (1 h at 37°C) in assembly buffer containing $C_{18}H_{37}SO_4Na$ inducer (50 μ M) and either (*top*) mixed histones or (*bottom*) httau40, subjected to absorbance spectroscopy, then plotted as extinction coefficient (ϵ ; normalized to monomer band intensity in methanol) versus wavelength. The positions of N744 absorbance bands corresponding to monomer (M), dimer (D), H-aggregate (H), and J-aggregate (J) are shown at the top of each panel. The dotted line in the top panel depicts the absorbance of monomeric N744 in neat methanol solvent. See text for details. Note: ordinate scales differ in the two panels.

Figure 6. N744 dimerizes in the presence of tau

Concentrations of N744 in monomeric (C_m) and aggregate ($C_t - C_m$) forms were calculated after deconvolution of the absorbance spectra shown in Fig. 5. Data points correspond to spectra collected in the presence of either httau40 (●) or mixed histones (○), with the associated errors corresponding to the S.E. of the estimate of the Gaussian fit of spectral data. Each line represents best fit of the data points to a linear regression. In the presence of httau40, data points corresponding to the bulk N744 concentration range of 0.2 – 0.8 μM fell on a single regression line with slope 2.0 ± 0.1 . In the presence of histones, the regression slope was 2.4 ± 0.4 in the bulk N744 concentration range of 0.2 – 0.6 μM , but much higher in the range 0.6 – 1 μM (dotted line). These data indicate that dimerization is the principal aggregation reaction in the sub-micromolar concentration regime.

Figure 7. N744 modulates tau binding capacity of anionic inducer

Carboxylate-substituted polystyrene microspheres (124 pM) were incubated (24 h at 37°C) in assembly buffer containing htau40 (4 μM) and varying concentrations of N744. Aliquots were then visualized by transmission electron microscopy. **A**, In the absence of tau, microspheres appeared as smooth-surfaced, roughly spherical objects without any associated filamentous material. **B**, In the presence of tau protein, microspheres developed rough surfaces and appeared enlarged relative to beads incubated in the absence of protein, with 10 – 20% having associated filaments. The additional volume resulting from the increase in diameter (V_{shell}) was calculated using equation 4. **C**, increasing concentrations of N744 decreased the apparent shell volume, consistent with release of bound tau from the microsphere surface. Bar = 100 nm.