Testing the Therapeutic Potential of Doxycycline in a Drosophila melanogaster Model of Alzheimer Disease

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Background: We investigated doxycycline neuroprotection in a Drosophila model of AD.

Significance: Doxycycline is a potential therapeutic agent in AD.

Conclusion: Doxycycline prevents Aβ toxicity in vitro and in vivo.

Results: Doxycycline improves Aβ42 fly locomotion and partially rescues the toxicity of Aβ in the developing eye of E22G Aβ42 flies, preventing Aβ fibrillation and generating non-toxic structures.

Therapies for Alzheimer disease that reduce the production of pathogenic amyloid β (Aβ) peptides have been associated with a range of unwanted effects. For this reason, alternative strategies that promote the clearance of the peptide by preventing its aggregation and deposition in the brain have been favored. In this context we have studied doxycycline, a member of the tetracycline family of antibiotics that has shown neuroprotective effects in a number of models of neurodegenerative disease. We investigated the neuroprotective potential of doxycycline in a Drosophila model of Aβ toxicity and sought to correlate any effects with the aggregation state of the peptide. We found that administration of doxycycline to Aβ42-expressing flies did not improve their lifespan but was able to slow the progression of their locomotor deficits. We also measured the rough eye phenotype of transgenic flies expressing the E22G variant of Aβ42 and showed that doxycycline administration partially rescued the toxicity of Aβ in the developing eye. We correlated these in vivo effects with in vitro observations using transmission electron microscopy, dynamic light scattering, and thioflavin T binding. We found that doxycycline prevents Aβ fibrillation and favors the generation of smaller, non-amyloid structures that were nontoxic as determined by the lack of caspase 3 activation in a neuroblastoma cell line. Our confirmation that doxycycline can prevent amyloid β toxicity both in vitro and in vivo supports its therapeutic potential in AD.

Alzheimer disease (AD) is the most prevalent neurodegenerative disorder, affecting more than 35 million people worldwide. AD is histopathologically characterized by the presence of intracellular neurofibrillary tangles and extracellular senile plaques that become increasingly widespread within the brain as the disease progresses (1). Neurofibrillary tangles are composed of hyperphosphorylated τ, whereas senile plaques contain insoluble fibrillar aggregates of a polypeptide named amyloid β peptide (Aβ), which can also be found in the walls of blood vessels (2). Aβ is a normal, soluble product of neuronal metabolism (3, 4) and is secreted into the extracellular space following proteolytic cleavage of the amyloid precursor protein by the proteases β- and γ-secretase. The physicochemical features of Aβ, in particular its propensity to aggregate, depend on the precise site of the γ secretase cleavage. In health, the shorter and less aggregation-prone Aβ40 peptide predominates, whereas in disease, the more hydrophobic Aβ42 is overproduced (5). Data from familial AD cases indicate that the production of aggregation-prone isoforms of Aβ is sufficient to cause disease (6), and so therapeutic strategies have focused mainly on the prevention of the peptide generation (7–9). Unfortunately, the development of γ secretase inhibitors in particular has been hampered by a range of serious unwanted effects. Consequently, alternative interventions that accelerate the catabolism of Aβ are increasingly attractive, and in this regard, drugs that modulate the aggregation of Aβ or disrupt existing Aβ aggregates may be beneficial (10–13). The tetracyclines are a ubiquitous family of antibiotics that are safe in clinical practice, and some, including doxycycline and minocycline, are able to cross the blood-brain barrier (14). However, their therapeutic effects are not just antimicrobial. Indeed, there are reports of neuroprotective effects in several models of neurological disorders, including cerebral ischemia (15, 16), spinal cord injury, Parkinson’s disease (17, 18), Huntington’s disease (19–21), amyotrophic lateral sclerosis (22, 23), multiple sclero-

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sis, familial amyloidotic polyneuropathy (24, 25), and AD (26, 27).

Familial amyloidotic polyneuropathy is an autosomal dominant disorder that is characterized pathologically by the extracellular deposition of mutant transthyretin (TTR) fibrils throughout the peripheral nervous system. Cardoso et al. (24) used a murine model of familial amyloidotic polyneuropathy to determine the efficacy of several fibril-disrupting compounds, including tetracyclines. In vitro, doxycycline had the highest fibril-disruption activity, and in vivo studies using a mouse model of familial amyloidotic polyneuropathy also demonstrated a beneficial effect. Doxycycline was able to disrupt TTR amyloid deposits and decrease standard markers associated with the deposition of fibrils (25). More recently, combined administration of doxycycline and tauroursodeoxycholic acid was trialed in the same mouse model. Tauroursodeoxycholic acid is a non-toxic hydrophilic bile acid with antiapoptotic properties. Combination therapy, in the same mouse model, provided synergistic improvements in disease markers that report amyloid deposition and non-fibrillar TTR accumulation (28). On the basis of the in vivo data, a phase II preclinical trial, using this combined doxycycline/tauroursodeoxycholic acid administration, is ongoing in patients with TTR amyloidosis (identifier NCT01171859). Forloni et al. (29) were the first to show the neuroprotective properties of doxycycline in AD. Using electron microscopy (EM) and thioflavin T (Th T) fluorescence assays to assess Aβ aggregation, it has been shown that tetracyclines inhibit both aggregate formation and also disrupt preformed fibrils. In this work, we investigate the in vivo effects of doxycycline in a Drosophila melanogaster model of Aβ toxicity in AD and gain further insights into the molecular mechanism.

EXPERIMENTAL PROCEDURES

Generation and Characterization of Drosophila melanogaster-expressing Aβ Peptides—Transgenic flies carrying Upstream Activation Sequence (UAS) expression constructs for Aβ40 (Alz40.3), Aβ42 (Alz42.1, Alz42.2), and the E22G variant of Aβ42 (Arctic Aβ42) (AlzArc2) have been described previously (30). UAS-GFP were sourced from the Bloomington Stock Center. Flies carrying UAS-responsive constructs were crossed to driver lines that express gal4 in neurones (30). UAS-GFP were sourced from the Bloomington Stock Center. Control flies were generated by crossing the w1118 strain. All fly culture was performed at 25 °C.

Immunohistochemistry—Fly brains were isolated by dissection in PBS with 0.05% (v/v) Triton X-100 and fixed in 4% (w/v) paraformaldehyde for 1 h at room temperature. Brains were washed three times in PBS with 0.05% (v/v) Triton X-100 and blocked in 5% (w/v) bovine serum albumin for 1 h at room temperature. Mouse anti-Aβ (6E10, Signet), diluted 1:1000 in blocking buffer, was incubated with the tissue overnight. After three further washes, the tissue was incubated in goat anti-mouse IgG Alexa Fluor 546 (Invitrogen) and counterstained with TO-TO-3 (Invitrogen) before mounting in Vectashield (Vector Labs). Confocal serial scanning images were acquired at 2- or 4-µm intervals using a Nikon Eclipse C1si on a Nikon E90i upright stand (Nikon). The image stacks were projected using ImageJ (version 1.42k), and the final composite images were processed with Photoshop CS4 software (Adobe Systems).

Protein Extraction and Western Blotting—Twenty-five flies of each genotype were snap-frozen in liquid nitrogen and decapitated by vortexing. Fly heads were homogenized in PBS with 1% (w/v) SDS and protease inhibitors (Complete, Roche). The homogenate was centrifuged at 18,000 × g for 2 min, and the supernatant was removed and termed the “soluble Aβ fraction.” The respective pellets were then homogenized in urea (9 M urea, 1% (w/v) SDS, 25 mM Tris HCl, 1 mM EDTA), sonicated, and maintained at 55 °C for 1 h. Homogenates were centrifuged at 18,000 × g, and the supernatant was removed and termed the “insoluble Aβ fraction.” The protein concentration was determined by Detergent-Comparable (DC) Protein assay (Bio-Rad). Equal amounts of the protein were loaded on to 4–12% (w/v) acrylamide Bis/Tris SDS-PAGE gels (Invitrogen). Electrophoresis occurred under non-reducing conditions, and protein was semi-dry transferred to nitrocellulose membranes. Aβ was detected using a mouse monoclonal anti-Aβ antibody (6E10, Signet). All blots were developed using a Supersignal West Femto Maximum Sensitivity ECL substrate (Pierce).

Doxycycline Solutions Preparation—Doxycycline hyclate (Sigma) was dissolved in distilled water and filtered through a 0.2-µm filter (Orange Scientific), aliquotted at a range of concentrations (0, 20, 50, 100, and 360 µM) and stored at −20 °C in the dark. Flies were cultured on media prepared by mixing an equal weight of the required concentration of doxycycline with instant fly food powder (Phillips Scientific).

Survival Assays—Survival assays were performed as described by Crowther et al. (30). Briefly, 100 flies of each genotype were collected, divided into tubes of 10 flies, kept at 25 °C, and transferred to fresh food containing 0 µM, 20 µM, 50 µM, and 100 µM of doxycycline, every 2–3 days. The number of live flies and those lost to follow-up was counted every 2–3 days. Survival curves were analyzed using Kaplan-Meier plots and log-rank statistical analysis.

Climbing Assays—To assess climbing behavior, 10 flies expressing Aβ40, Aβ42 peptides and Gal4-elav1155 driver were placed at the bottom of a tall cylinder (25 ml tissue culture pipette) and allowed to climb for 30 s before the number of flies at the top and at the bottom was determined as described previously (31). A performance index was calculated over the climbing behavior of the cohort of flies from eclosion until day 36 of adult life. The experiment was repeated three times.

Rough Eye Phenotype Assessment—Control and Arctic Aβ42 flies were crossed with GMR-Gal4 flies to drive expression of the transgene in the developing eye. The flies were kept throughout their development at 25 °C on food containing between 0 and 100 µM doxycycline, and the offspring were kept at the same temperature. Flies were snap-frozen over dry ice/ethanol on days 1 and 5 post-eclosion, and the structure of their compound eyes was recorded using a light microscope Nikon SMW-U equipped with Nikon digital camera (3.7 megapixels). Photographs of the eyes of the flies were taken with identical microscope settings. ImageJ was used to estimate the area of each eye. To quantify the ommatidia, we determined a particular part of the eye where we could easily count the ommatidia and then estimated its area. The number of ommatidia was
divided by the selected area, and it is presented. The area of each eye and the number of ommatidia was expressed as a ratio of the size of control fly eyes, and a total of four eyes per condition were analyzed.

Thioflavin T Binding Assay and Transmission Electron Microscopy—Aggregation of the Aβ42 peptide was measured by Th T binding assay. Aβ was dissolved at 50 μM in 50 mM Tris HCl (pH 7.5) and incubated at 37 °C with 100 μM and 360 μM of doxycycline for different time points (0 and 5 days). Excitation spectra were recorded on a Horiba Fluoromax spectrofluorometer at 25 °C with 30 μM Th T (Fluka) in 50 mM glycine/NaOH buffer at pH 9.0 in a 1-ml assay volume. Results were presented by plotting the fluorescence intensity at 450 nm, the characteristic excitation maxima formed upon Th T binding. Experiments were repeated at least three times.

For visualization by TEM, sample aliquots (10 μl) were adsorbed onto glow-discharged, carbon-coated collotion film supported on 200-mesh copper grids and negatively stained with 1% (w/v) uranyl acetate. Grids were visualized with a Zeiss microscope (model EM10C) operated at 60 kV. The experiment was repeated three times.

Dynamic Light-scattering (DLS) Measurements—The size of the generated species in the presence or absence of doxycycline was measured at 633 nm on a dynamic light-scattering instrument (Zetasizer Nano ZS, Malvern Instrument). All measurements were made at 25 °C with a detection angle of 173°. Fifty μM Aβ42 in 50 mM Tris HCl (pH7.5) was incubated either with 100 μM or 360 μM doxycycline (prepared in distilled water) and the particle sizes were analyzed at different time points (0 and 5 days). The intensity of size distribution was obtained from the analysis of the correlation function using the multiple narrow mode algorithm of the Malvern DTS software. The experiment was repeated three times.

Cell Culture and Caspase 3 Assay—SH-SY5Y cells (a human neuroblastoma cell line) were propagated in 6-well plates and maintained at 37 °C in a 95% (v/v) humidified atmosphere and 5% (v/v) CO2. The culture medium consisted of Dulbecco’s minimal essential medium with Ham’s F12 (1:1) supplemented with 1% (w/v) non-essential amino acids (Invitrogen), 2 mM l-glutamine, 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Lonza). Activation of caspase 3 was measured using the CaspACE fluorimetric 96-well plate assay system (Sigma) following the manufacturer’s instructions. Briefly, 10 μM Aβ in Ham’s F12 cell medium was preincubated alone or in the presence of 10 μM doxycycline (in distilled water) at 37 °C for 5 days were added to 80% confluent cells in medium without FBS and further incubated for 24 h. Two control conditions were also tested. These were doxycycline incubated with cells in the absence of Aβ and doxycycline spiked into preaggregated Aβ just prior to addition to the cells. Subsequently each well was trypsinized, and the cell pellet was lysed in 100 μl of hypotonic lysis buffer (Sigma). Forty μl of each cell lysate was then used in duplicates for determination of caspase 3 activation. The remaining cell lysate was used to measure total cell protein concentration with the Bio-Rad protein assay kit using bovine serum albumin as the protein standard. The data shown is the mean of duplicates, and the experiment was performed three times.

Statistics—Statistical analyses were performed using GraphPad Prism 4.0 for Macintosh. Results are reported as mean ± S.D. Statistical analysis was performed by Students t test, one-way analysis of variance and two-way analysis of variance followed by Tukey’s post hoc test and Bonferroni post hoc test, respectively.

RESULTS

Aβ Expression in D. melanogaster—Flies expressing Aβ42 and Arctic Aβ42 in neurones have been shown to accumulate toxic non-amyloid aggregates, firstly intracellularly and then extracellularly, causing neuronal dysfunction and then neurodegeneration (30). Fig. 1 (left panel) depicts Aβ expression in the brain of the various flies analyzed immediately after hatching (day 0). Confocal microscopy showed diffuse peptide aggregates (red) distributed throughout the brain of Aβ42 flies, which are absent in a control brain (Fig. 1A, right panel). This result was confirmed by Western blot using the 6E10 antibody that revealed the existence of soluble and aggregated forms of the peptide (Fig. 1B), both in flies grown in the absence and in the presence of a high doxycycline concentration, showing that the drug does not interfere with Aβ expression. Transcription of the Aβ40 transgene in the flies is equivalent to the Aβ42 (32). However, the lower aggregation propensity of the shorter peptide isoforms results in less peptide accumulation (Fig. 1B), most likely because of faster clearance.

Longevity of Aβ42 Flies Is Not Affected by Doxycycline—Flies expressing Aβ in their nervous system and control flies were exposed to a range of doxycycline concentrations in the culture medium (0, 20, 50, and 100 μM), throughout development and adulthood.

Although control and Aβ40 flies had a median survival of 78 days, in contrast, flies expressing Aβ42 died sooner, with a median lifespan of 47 days (Fig. 2, n = 100, p < 0.001). The addition of doxycycline at a range of concentrations had no effect on the longevity of any of the fly lines mentioned above. Moreover, there was no effect of drug treatment on the longevity of an independent line of flies expressing GFP (Fig. 2).

Doxycycline Rescues the Locomotor Deficits Seen in Aβ42-expressing Flies—A reduction in longevity is, by definition, the latest phenotype that can be measured in our model system, and so we turned to locomotor assays to detect earlier AD-related neuronal dysfunction. Specifically, we performed climbing assays to determine whether doxycycline could delay the accelerated age-related decline in locomotor performance that is associated with Aβ42 expression.

Control flies and those expressing Aβ40 and GFP flies exhibited the expected gradual age-related reduction in climbing behavior (performance indices falling from 85% at day 5 through 70% at day 22 to 30% at day 36, Fig. 3 and supplemental Fig. S1). In contrast, flies expressing Aβ42 exhibited a much earlier and more rapid locomotor decline (performance indices falling from 85% at day 5 through 20% at day 15 to zero after day 26, Fig. 3 and supplemental Fig. S1). Treating control flies and those expressing Aβ40 and GFP with different doxycycline concentrations had no effect on their climbing abilities at any age (Fig. 3 and supplemental Fig. S1). In contrast, doxycycline rescued the locomotor performance of flies expressing Aβ42,
increasing their climbing index to 40% at day 15 for the lowest drug concentration used (20 μM). This effect was drug concentration-dependent, with optimal results observed at 50 μM doxycycline, at which concentration there was complete rescue of the Aβ-induced reduction in locomotor activity at day 15 (Fig. 3). Thereafter, the neuroprotective effects were maintained until day 19 (supplemental Fig. S1).

**Doxycycline Partially Rescues the Aβ Peptide Rough Eye Phenotype—**D. melanogaster has a compound eye containing an array of about 800 ommatidia. The normal regular array of ommatidia may be disturbed by driving toxic transgenes with GMR-Gal4 (35, 36). Expression of Aβ42 in Drosophila retinal tissue at 29 °C produces a mild rough eye phenotype along with associated reduction in eye size, as described previously (30). Although less intense, this phenotype is also observed at 25 °C. The expression of Arctic Aβ42 causes a more marked phenotype (30) and permitted us to screen for rescue by 100 μM doxycycline or vehicle alone.

Control flies showed no change in the size of their eyes over time, either in the presence or absence of doxycycline (Fig. 4A, upper panels, and B). By contrast, doxycycline partially rescued the Arctic Aβ42-induced reduction in eye size that is seen in flies at days 0 and 5 (Fig. 4A, lower panels, and B).

With regard to ommatidial irregularity, control flies exhibited a mild phenotype (Fig. 4C), indicating that Gal4 itself is slightly toxic during eye development. The more marked irregularities seen in Aβ42-expressing flies were partially rescued by treatment with doxycycline at day 0 (Fig. 4A, lower left panels,
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FIGURE 3. Doxycycline improves locomotion performance of Aβ42 flies. Performance index (climbing %) of control, Aβ40, GFP, and Aβ42 flies at days 5 (light gray bars) and 15 (black bars). The locomotion impairment observed for Aβ42 flies after day 5 is completely rescued by 50 μM doxycycline treatment. ***, p < 0.001 compared with the same concentration at day 5; ###, p < 0.001 compared with concentration 0 μM for the period of 15 days; &&, p < 0.01 compared with concentrations 20 μM for the period of 15 days.

and C). At day 5, no significant differences were observed between the treated and untreated Arctic Aβ42 flies, although a trend was observed (Fig. 4A, lower right panels, and C), suggesting that Aβ accumulation overcomes doxycycline capacity to protect against the harmful effects of the peptide.

Doxycycline Reduces the Aggregation of Aβ42 Peptide in Vitro—The effect of doxycycline on Aβ42 aggregation was assessed by Th T fluorescence, a method that specifically reports the presence of β-sheet-containing structures (37, 38), at two time points. At day 0, 50 μM Aβ42 alone or incubated with 100 μM and 360 μM of doxycycline, failed to show a Th T fluorescence signal (Fig. 5A). However, at day 5, Aβ42 peptide showed an increase in the fluorescence intensity, which was progressively reduced when the peptide was coincubated with 100 μM and 360 μM of drug (Fig. 5A). We excluded the possibility that doxycycline and Th T compete for the same binding site on Aβ by performing an extra control (the asterisk in Fig. 5A) where we allowed the peptide to aggregate to completion in the absence of doxycycline and then spiked the aggregates with 360 μM doxycycline just before the Th T fluorescence measurement.

When the same Aβ samples were visualized using TEM, control reactions showed small, round Aβ oligomers at day 0 (Fig. 5B, top left panel), whereas by day 5, long, unbranched, 10-nm-diameter fibrils were the most predominant species. No oligomers, aggregates, or other intermediate species were detected at this time point (Fig. 5B, left bottom panel). Preparations of Aβ preincubated with 100 μM doxycycline for 5 days generated thinner and shorter fibrils as compared with Aβ alone but no small aggregates or oligomers were visible (Fig. 5B, bottom middle panel). The effect of the drug was dose-dependent. Increasing its concentration to 360 μM resulted in the formation of small structures similar to ones found at the beginning of the incubation time. The presence of fibrils was scarce, and those few detected were also thinner and shorter than the ones found in control preparations (Fig. 5B, right bottom panel).

When the same peptide preparations were studied further using DLS (Fig. 5C), we observed that the progression in particle size (radius - r, nm) with time differed according to whether doxycycline was present or not. Initially, Aβ presented as a heterogeneous preparation with radii from 10–100 nm. After 5 days at 37 °C, the sample was composed of a homogeneous population of species with radii of about 2000 nm (Fig. 5C, comparison between days 0 and 5). The presence of 100 μM doxycycline for 5 days resulted in smaller aggregates, ranging from 1200–1900 nm (Fig. 5C, center right panel). Aβ peptide treated with 360 μM doxycycline, also for 5 days, dramatically reduced the size of the generated species (Fig. 5C, bottom right panel), resulting in two populations, with rare species ranging from 8–20 nm and a more abundant population in the range of 150–840 nm.

Doxycycline Prevents Aβ42-inherent Toxicity in Cell Culture—Although doxycycline is able to prevent Aβ aggregation as shown here, the toxic potential of the reaction products is not known. We measured the toxicity of the newly generated Aβ species by assessing apoptosis using a caspase 3 activation assay in a culture of SH-SY5Y cells. When comparing caspase 3 activation following Aβ42 treatment, we found that the presence of doxycycline during the aggregation of Aβ reduced apoptosis (Fig. 6). To exclude the possibility that the protective action of doxycycline occurs via other mechanisms besides its interference with Aβ fibrillogenesis, we evaluated the effect of the drug added to preaggregated Aβ just prior to its addition to cells, showing that the peptide was still toxic and able to induce caspase 3 activation (Fig. 6, +).
DISCUSSION

We have used a convenient model of AD to probe various therapeutic strategies that might reduce Aβ toxicity. Many existing compounds aim to reduce Aβ production by blocking β or γ secretase or by stimulating α secretase cleavage of amyloid precursor protein. Recent studies have shown that inhibitors of β and blockers of γ secretase are very hard to prepare and may cause side effects, respectively, because they are important for the cleavage of other molecules (41). Alternatively, stimulation of the non-amyloidogenic amyloid precursor processing is being developed (8). The peptide and its precursor are normal products of the cells, and although their physiological roles in neuronal function are still unknown, the implication in neuronal and synaptic activity has been suggested (4). Thus, control of physiological levels rather than complete inhibition seems to be a good option to reduce the accumulation of the peptide and to retard the progression of AD. Several molecules have been described that may be able to favor the catabolism of Aβ and its clearance from the brain. Members of the widely used tetracycline class of antibiotics have been proposed to have antiaggregation, fibril-disrupting, and cytoprotective effects. Among the known tetracyclines, doxycycline has been the favored drug used because of its pharmacokinetic advantages, namely a long plasma half-life making twice-a-day dosing possible and excellent penetration of the blood-brain barrier (42). Tetracyclines are multipotent molecules (33, 34) and their role in neurodegenerative disorders was described recently. Thus, antiamyloidogenic activity was already described in vitro for prion protein and Aβ42 and in vivo for TTR (24, 25, 29, 43). The benefits of doxycycline have not yet been established in vivo in AD. Therefore, the principal aim of this study was to determine the effects of doxycycline in a Drosophila model of Aβ toxicity and to evaluate its use as a potential therapeutic agent.

In this work, doxycycline was administered to the developing and adult Drosophila in their culture medium. The effect of the drug on the behavior of the flies was measured in three ways. Firstly by measuring the life span, secondly be assessing their climbing ability, and at thirdly by looking for effects on a developmental retinal toxicity phenotype (the rough eye phenotype). The drug had no effect on the longevity of the flies. In contrast, 50 and 100 μM doxycycline were able to rescue the rough eye phenotype given by the Arctic Aβ42 expression. **, p < 0.01 compared with 0 μM at day 0; ###, p < 0.001 compared with 0 μM at day 5; + +, p < 0.01 compared with 100 μM at day 0. C, quantification of ommatidia in control and in Arctic Aβ42 flies, untreated and treated with doxycycline 100 μM, at days 0 and 5, showing that the drug was able to promote partial recovery. **, p < 0.01 compared with 0 μM at day 0; ##, p < 0.1 compared with 100 μM at day 0; ##, p < 0.01 compared with 100 μM at day 0. ***, p < 0.001 compared with 0 μM at days 0 and 5; ###, p < 0.01 compared with 100 μM at day 0.
climbing behavior of the Aβ42-expressing flies during the first 19 days of adult life. At the highest tested concentration (100 μM), doxycycline was also able to partially rescue the rough eye phenotype resulting from the expression of the arctic Aβ42 peptide. Doxycycline appeared to delay disease progression and consequently improved the quality, if not the quantity, of life for the flies. The failure to prolong life span may be due to the reduction in feeding behavior that is observed with increasing age. This would predictably result in clearance of the drug in older flies just when peak levels of Aβ have accumulated. Accordingly, locomotor improvement was observed during the first 19 days of life, but differences in life span (between controls and Aβ42) were only evident much later, around day 45. Similarly, ommatidial irregularity was recovered by doxycycline treatment upon eclosion, but this effect was not maintained over time, and at day 5, although a trend was observed, the

FIGURE 5. Doxycycline impedes the conformational progression of the Aβ42 peptide. A, ThT binding assay of 50 μM of synthetic Aβ42 incubated alone or with different concentrations of doxycycline (0, 100, and 360 μM), showing the ability of the drug to decrease the amyloidogenic potential of Aβ42. Doxycycline does not interfere with the assay as confirmed by its addition at 360 μM to Aβ42 just prior to fluorescence measurement (360*) *** p < 0.001 compared with 0 μM and 360* μM for the period of 5 days; ##, p < 0.01 compared with 100 μM for the period of 5 days. B, TEM analyses of Aβ42 alone or in the presence of 100 μM and 360 μM doxycycline at 0 (upper panels) and 5 (lower panels) days, showing that the drug was able to inhibit Aβ fibrillogenesis in a dose-dependent manner. Scale bar = 200 nm. C, DLS analyses of Aβ42 alone (top panels) or incubated with 100 (center panels) and 360 μM (bottom panels) of doxycycline at 0 (left panels) and 5 days (right panels), showing the ability of the drug to reduce the size of Aβ-formed particles.
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Doxycycline 10 μM

![Graph showing Caspase-3 activation and Fluorescent/Total protein content (mg/mL) for different conditions.]

**FIGURE 6. Doxycycline prevents Aβ inherent toxicity.** 10 μM of Aβ42 alone or in the presence of 10 μM of doxycycline incubated at 37 °C for 5 days were added to SH-SY5Y cultured cells and further incubated for 24 h at 37 °C, showing that Aβ42 species generated upon doxycycline treatment were significantly less toxic than species of Aβ alone. Doxycycline added to aggregated peptide just prior to its addition to cells did not protect against Aβ toxicity. ***, p < 0.001 and **, p < 0.01 compared with untreated cells; #, p < 0.05 compared with cells treated with preaggregated Aβ and doxycycline 10 μM added just prior to addition to cells.

recovery was not as evident, supporting the notion that the Aβ load surpassed the initial protection by doxycycline.

Similar doxycycline concentrations were tested in other AD animal models and found to be protective, for example in Caenorhabditis elegans (44). In this study, we observed rescue of the expected paralysis phenotype in Aβ42-expressing CL4176 worms when they were treated with 50 μM doxycycline and other tetracycline derivatives (44). Faust et al. (45), using a Parkinson’s disease fly model, demonstrated that 50 and 100 of μM minocycline were able to protect dopaminergic neurons against degeneration, thus preventing and/or delaying the progression of Parkinson’s disease. Whether doxycycline is beneficial in patients with AD is not yet clear. However, a trial of daily dosing with doxycycline and rifampin for 3 months preceding the MALDI-TOF MS analysis.

In this work we have studied whether the protective effects seen for doxycycline in our Drosophila model may be mediated by modulating the aggregation of the Aβ peptide. Our results show that in vitro doxycycline reduces formation of amyloid aggregates, the number of fibrils, and the size of Aβ species formed using Th T fluorescence, by TEM and DLS analysis. The tetracyclines contain a hydrophobic core formed by aromatic moieties containing hydrophilic substituents conferring an amphiphilic character. Strong interactions between these molecules and the lipophilic domains of misfolded proteins can be observed. Airoldi et al. (14) found that the doxycycline-Aβ42 interaction involved all the drug protons, but without a specific binding site, and suggested the presence of a supramolecular interaction, which would prevent fibril formation. Other authors suggested that doxycycline decreases the resistance of fibrils to proteolysis (29). However, the putative mechanism is not clear.

Future work should also address the influence of doxycycline on the in vivo aggregation state of Aβ in the fly brain to further clarify the results presented here. It is now widely accepted that oligomers and other initial species are the toxic elements and that mature fibrils cause little or no toxicity to cells (46, 47). One of the concerns when designing therapies that inhibit or disrupt amyloid is that the newly stabilized aggregation intermediates will themselves be toxic. Thus, care should be taken when selecting antiamyloid compounds, and their cellular effects should be evaluated carefully. For this reason we measured the cytotoxicity of Aβ and found that in the presence of doxycycline there was a significant reduction in caspase 3 activation when compared with peptide alone. Although this signal of apoptotic cell death in the presence of doxycycline was significantly reduced it was not as low as in untreated cells. This could be a consequence of direct doxycycline toxicity, or else it may indicate that a very small percentage of the Aβ species that are stabilized by the drug might produce some toxicity.

Taken together, our data indicate that doxycycline modulates Aβ aggregation and that in an in vivo model it can provide a specific neuroprotective effect. Our results provide support for the trialing of doxycycline as a potential therapeutic agent in AD.

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