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Methylene blue does not reverse existing neurofibrillary tangle pathology in the rTg4510 mouse model of tauopathy

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Abstract

Alzheimer's disease is characterized pathologically by aggregation of amyloid beta into senile plaques and aggregation of pathologically modified tau into neurofibrillary tangles. While changes in amyloid processing are strongly implicated in disease initiation, the recent failure of amyloid-based therapies has highlighted the importance of tau as a therapeutic target. "Tangle busting" compounds including methylene blue and analogous molecules are currently being evaluated as therapeutics in Alzheimer's disease. Previous studies indicated that methylene blue can reverse tau aggregation in vitro after 10 minutes, and subsequent studies suggested that high levels of drug reduce tau protein levels (assessed biochemically) in vivo. Here, we tested whether methylene blue could remove established neurofibrillary tangles in the rTg4510 model of tauopathy, which develops robust tangle pathology. We find that 6 weeks of methylene blue dosing in the water from 16 months to 17.5 months of age decreases soluble tau but does not remove sarkosyl insoluble tau, or histologically defined PHF1 or Gallyas positive tangle pathology. These data indicate that methylene blue treatment will likely not rapidly reverse existing tangle pathology.

Keywords

Alzheimer; tau; methylene blue

Introduction

Alzheimer's dementia is a devastating condition for which there are currently no effective treatments. Genetic evidence from rare familial cases of Alzheimer's indicate that altered amyloid processing is central to disease pathogenesis, but amyloid pathology in the brain

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does not correlate well with cognitive decline [6] and there have been several recent failures in amyloid-directed therapeutic trials [9]. In contrast, tau pathology in the form of neurofibrillary tangles parallels synapse loss, neuronal loss, and dementia, leading to the idea that tangles are neurotoxic and that reversal of tangles would be therapeutic. Thus therapeutic strategies to dissociate tangles have become of interest. Several tau-directed strategies have been developed including immunotherapy, chaperone-based protein degradation, and inhibitors of aggregation. Wischik et al reported in 1996 that methylene blue, a phenothiazine compound, inhibits tau aggregation and can dissociate paired-helical filaments in vitro [18]. Phenothiazines are of interest as they are bioavailable and have in the past been used to treat several conditions including methemoglobinemia, schizophrenia and anxiety with few adverse effects [10, 13, 15]. The first anti-tangle therapy in humans was based on this work and described at the International Conference on Alzheimer's Disease by Wischik in 2008, in which phase II clinical trial data was presented with reported improvements in some patients taking methylene blue.

On the basis of this potentially exciting data, preclinical studies have been performed in animal models to test the hypothesis that methylene blue can ameliorate tau-related neurodegeneration. Treatment of 3 month-old rTg4510 mice for 12 weeks with oral methylene blue prevented behavioral deficits and reduced soluble tau levels in the brain [11]. JNPL3 mice treated with methylene blue for 2 weeks similarly showed reductions in soluble tau levels without affecting insoluble tau levels [2]. These studies indicate that methylene blue treatment can reduce soluble tau levels and prevent cognitive decline when treatment begins at a time point before neurofibrillary tangles are present in the brain [11]. However, it was not previously known whether methylene blue can dissolve existing neurofibrillary tangles, its putative mechanism of action. To test this hypothesis, we treated rTg4510 mice with advanced neurofibrillary pathology with methylene blue for six weeks. We find that contrary to in vitro findings, methylene blue does not appear to dissociate neurofibrillary tangles in the mouse brain.

Materials and Methods

Animals and drug treatment

rTg4510 mice express human P301L mutant tau under the control of a tetracycline-operon-responsive element and an activator transgene consisting of a tet-off open reading frame downstream of calcium calmodulin kinase II promoter elements [14]. Mice used were mixed genders of F1 progeny crosses between the activator transgene on a 129 background strain and the tau responder transgene on an FVB background (n=5 methylene blue treated, 5 vehicle treated) and littermates expressing only the activator transgene (n=5 methylene blue treated, 5 vehicle treated). Mice were treated from 16 months of age to 17.5 months of age with either methylene blue (blue, not colorless form at 0.062 mg/mL = 166 μ M in 2mM saccharine) or saccharine vehicle alone in the drinking water. As published previously, this results in an estimated dose of 9.3mg/kg/day of methylene blue [11]. At the end of treatment, mice were sacrificed by CO₂ inhalation and perfused transcardially with 0.01M phosphate buffered saline to remove blood from the brain. Brains were removed and one hemisphere fixed in 4% paraformaldehyde for 48hours and the other hemisphere snap frozen

for analysis of drug penetration into the brain. Animal studies were conducted in accordance with NIH and institutional animal care guidelines. Approval for animal experiments was gained through the Subcommittee on Research Animal Care at the Massachusetts General Hospital approval 2004N000092 and experiments involving mice were reviewed and approved by McLaughlin Research Institute's Institutional Animal Care and Use Committee under protocol GAC-06. MRI's Assurance number with the NIH's Office of Animal Welfare is A3901-01. Drug treatments were dissolved in drinking water and had no adverse effects on the animals. Euthanasia was carried out by approved methods and all efforts were made to minimize suffering.

Assessment of drug levels in the brain

The amount of methylene blue in the brains of 5 methylene blue treated and 5 vehicle treated animals was determined by liquid chromatography (LC) and mass spectrometry (MS) at Apredica (Watertown, MA) using the following procedures. Mouse brain samples were thawed on ice and kept at 4 °C during processing. Brain tissues were homogenized in equal volume of PBS, pH 7.4. An aliquot brain homogenate sample or calibration sample were mixed with three volumes of methanol containing internal standard, incubated on ice for 5 min, and centrifuged. The protein-free supernatant was used for analysis. A working dilution of methylene blue in DMSO at 50 times the final concentration was prepared and serially diluted samples were prepared. These samples were diluted 50-fold into mouse blank brain homogenate and mixed with three volumes of methanol containing internal standard, incubated on ice for 5 min, and centrifuged. Samples were analyzed by LC/MS/MS using an Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent). After separation on a C18 reverse phase HPLC column using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS).

Histology and stereology

Fixed brain hemispheres were cryoprotected in 15% glycerol then cut into 50 µm coronal sections. Every 10th section was immunostained for hyperphosphorylated tau (PHF1 primary antibody, courtesy Dr Peter Davies) and an HRP-conjugated anti-mouse secondary antibody (Jackson Immuno Research) visualized with diaminobenzidine staining (Vector Laboratories). Nuclei were counterstained with cresyl violet. Another series of sections was labeled with Gallyas silver staining as previously described [4]. PHF1 positive neurofibrillary tangle numbers and neuron numbers were estimated in the CA1 region of the hippocampus and in the neocortex using optical disector stereology on an Olympus upright microscope equipped with a CAST stereology system (Olympus Denmark) as described previously [16]. Briefly, the region of interest was outlined on every 10th section at low magnification and the area measured. Counting frames of 21.8×21.8 mm were placed in a systematic random fashion throughout the CA1 and neocortex. Neurons identified by nuclear morphology and PHF1 positive neurons were counted in the counting frames and the density of each calculated by dividing the number counted by the combined volume of all counting frames. Volumes of the entire CA1 and neocortex were calculated with the Cavalieri method and the density of neurons and PHF1 positive neurons were multiplied by

the region volume to estimate the total number of neurons and PHF1 positive neurons per hemisphere.

To confirm that PHF1 positive neuronal counts reflected mature tangle pathology, Gallyas staining was assessed in CA1 on a single section per brain at Bregma -2.0mm. All Gallyas positive tangles in the CA1 were counted in this single section per brain.

Assay of soluble and insoluble tau levels in the brain

rTg4510 mice treated with methylene blue (n=5) or vehicle (n=5) were sacrificed by CO₂ inhalation and brains were dissected and snap frozen on dry ice. Purification of sarkosyl-insoluble tau was performed as described previously [3, 5]. Briefly, 45 µg of forebrain from each mouse was homogenized in buffer H (10mM Tris-HCl, pH 7.5 containing 0.8M NaCl, 1 mM EGTA, and 1mM DTT) and spun at 100,000×g for 20 min at 4°C. The supernatant was collected as the TBS soluble fraction. A further 2 mL of buffer H was used to resuspend the pellet in a polytron. Samples were then incubated in 1% Triton-X100 at 37°C for 30 minutes, centrifuged at 100,000 ×g for a further 20 min at 4°C, resuspended in 1 mL of buffer H, incubated in 1% sarkosyl for 30 min, and spun again at 100,000 ×g at 4°C for 30 min. The detergent-insoluble pellet extracted in 100 µL urea buffer (8 M Urea, 50 nM Tris-HCl, pH 7.5), sonicated, and spun at 100,000 ×g for 20 minutes at 4°C. The supernatant was collected (sarkosyl-insoluble fraction). Protein concentrations were determined by BCA assay and run on SDS-PAGE mini NuPAGE 4-12% bis-tris gels. Blots were probed with anti human tau tau13 antibody and infrared secondary antibodies and detected on a LiCor imaging system. Densitometry of blots was analyzed using ImageJ. For TBS soluble tau, the following number of animals were analyzed: n=5 vehicle treated rTg4510, 5 methylene blue treated rTg4510, 2 wild-type methylene blue treated, and 2 wild-type vehicle treated. TBS soluble tau levels were normalized to actin loading control. For sarkosyl insoluble extracts, the following number of animals were analyzed: n=4 vehicle treated rTg4510, 4 methylene blue treated rTg4510, 2 wild-type methylene blue treated, and 2 wild-type vehicle treated. Equal amounts of protein were loaded on the gels and the amount of sarkosyl insoluble tau in methylene blue treated animals was normalized to the amount found in vehicle treated animals as the detergent extraction prevents use of a housekeeping loading control. As expected, human tau was not detected in TBS or sarkosyl insoluble fractions in any wild-type animals.

Statistics

Normality of data was assessed using Shapiro-Wilkes tests. Since the stereology data within treatment groups and genotypes were not normally distributed, non-parametric tests were used to compare means (Kruskal-Wallis for multiple groups, post-hoc Wilcoxon tests to compare two groups). Significance was defined as $p < 0.05$. Data are presented as mean $\pm$ standard deviation of the mean. Western blot data quantifying human tau in rTg4510 mice were analyzed using t-test to compare vehicle and methylene blue treatments.

Results

rTg4510 animals and wild-type littermate control animals were treated with methylene blue or vehicle in the drinking water for six weeks. Penetration of drug into the brain was assessed by LC/MS/MS analysis revealing an average of 41 ± 25 nM methylene blue in the brains of 3 wild-type and 4 rTg4510 mice at the end of the treatment. There was no significant difference between the levels of methylene blue in the brains of treated wild-type and rTg4510 mice. Methylene blue was not detected in the brains of vehicle treated mice as expected.

At this advanced age (over 17 months), rTg4510 mice have substantial neurofibrillary pathology in the cortex and hippocampal formation [16]. Micrographs of PHF1 immunostaining and Gallyas silver staining (figure 1) demonstrate the neurofibrillary tangle pathology and brain atrophy evident at this late stage in our cohort of mice. We tested whether oral administration of methylene blue could reverse this pathology. Stereological estimates reveal that in vehicle treated animals, 24% of remaining CA1 neurons and 21% of remaining cortical neurons contain PHF1 positive tau inclusions with a total of $1.6 \times 10^4 \pm 1.0 \times 10^4$ PHF1 positive neurons in CA1 and $1.0 \times 10^6 \pm 0.2 \times 10^6$ PHF1 positive neurons in cortex. There is significant atrophy in both CA1 (46% volume reduction) and neocortex (32% volume reduction) of rTg4510 mice compared to wild-type littermates at this age. Neuronal counts reveal 53% neuronal loss in CA1 and 42% neuronal loss in neocortex in these vehicle treated rTg4510 mice compared to wild-type littermates (significant loss in both regions $p < 0.05$ Wilcoxon test compared to wild-type vehicle treated animals).

Treatment with methylene blue for six weeks did not reverse the neurofibrillary tangle pathology at this advanced stage. 20% of remaining CA1 neurons 21% of remaining neocortical neurons contained PHF1 positive tau inclusions after methylene blue treatment, which was not significantly different from the vehicle treated rTg4510 cohort. Estimates of the total number of PHF1 positive neurons in methylene blue treated mice were $1.1 \times 10^4 \pm 0.4 \times 10^4$ PHF1 positive neurons in CA1 and $1.2 \times 10^6 \pm 0.4 \times 10^6$ PHF1 positive neurons in cortex. To confirm that the PHF1 positive neuron data reflected mature neurofibrillary tangle pathology as has been previously published [16], Gallyas staining was performed and Gallyas positive neurofibrillary tangles counted in the CA1. No differences were observed in Gallyas staining with methylene blue treatment (29 ± 12 Gallyas positive cells in vehicle treated and 26 ± 7 in methylene blue treated animals per CA1 in a single section at Bregma -2.0, $p > 0.05$ Wilcoxon). Neuronal loss was also not affected by methylene blue treatment as there were no significant differences in total neuron number per hemisphere in vehicle vs methylene blue treated rTg4510 mice in either region. Similarly, volume loss was not reversed by methylene blue treatment. Figure 2 shows stereology quantification for CA1 and figure 3 for neocortex. In accord with the stereology data showing a persistence of tangle pathology, biochemical analysis revealed no reduction in sarkosyl-insoluble tau with methylene blue treatment (figure 4). As observed in previous studies, methylene blue treatment did significantly reduce the amount of TBS soluble tau in the brain ($p < 0.00001$, figure 4).

Together these anatomical and biochemical data from animals treated for 6 weeks with methylene blue at an advanced stage when substantial pathological lesions are present indicate that methylene blue does not reverse tau aggregation or rescue neuronal loss.

Discussion

Methylene blue is known to prevent and reverse tau fibrilization in vitro, even with only a few hours incubation with tau fibrils [17, 18], and clinical trials have been started based on the premise that this is a tangle-disintegrating drug. Two concerns, however, are whether methylene blue can reverse neurofibrillary lesions in vivo, and whether doing so would be therapeutically helpful in terms of prevention of neurodegeneration. Recent evidence has also suggested that soluble forms of tau, including oligomers, are more toxic to the brain than the aggregates themselves [7], which would urge caution in terms of removing tangles if the removal resulted in higher levels of the toxic soluble tau species. Preclinical studies using methylene blue in two different mouse models of tauopathy suggest that this drug actually reduces levels of soluble tau [2, 11]. It was previously not known whether methylene blue also clears existing neurofibrillary tangles or prevents neurodegeneration. While O'Leary et al assessed Gallyas positive staining semi-quantitatively (with % area stained) in younger animals (7 months of age) [11], this is the first study to examine stereologically whether methylene blue can reverse existing tangle pathology.

Here we find that, in the dose and time course examined, methylene blue does not clear existing tau pathology in neurons in the rTg4510 mouse model of tauopathy. Both PHF1 immunolabeled neurons and Gallyas positive tangles were unchanged with six weeks of treatment, but levels of TBS soluble tau were reduced by 35%. In a recent study using doxycycline to suppress tau transgene expression in rTgTauEC mice, we observed that neurofibrillary tangles can be cleared within 6 months of this substantial (>80%) reduction in soluble tau levels [12], indicating that perhaps long term treatment with methylene blue would reverse tangle pathology because it reduces soluble tau levels. However, based on the 35% reduction in soluble tau levels seen here and the data from our previous study, we estimate at least 18 months of treatment in mice would be required to reduce tangles if the effects of lowering soluble tau levels are assumed to be linear. This is over half of the lifetime of the mice, so this may not be a practical method to reduce tangles in people. However, reducing soluble tau may also have therapeutic benefits as soluble tau appears more toxic than tangles.

We also examined neuronal number in mice after methylene blue treatment. At this late stage (17.5 months), there is significant neuronal loss in the CA1 and cortex, which is not altered by six weeks of methylene blue treatment. Prevention of neuronal loss has, however, been indicated at younger ages in these mice with methylene blue treatment [11]. One limitation is that our study may have been underpowered to detect modest neuroprotective changes.

One possible explanation for the negative results found here is that methylene blue levels were not high enough in the brain to dissolve tangles. However, we observed brain penetrance of methylene blue in the nanomolar range (~40nM or 1.5 µg/100mg brain tissue)

after treating with 165 μ M methylene blue in the drinking water. This is very similar to the 0.7 μ g/100mg tissue observed by Congdon et al after treating mice with 0.2mg MB/kg by daily oral gavage [2]. In that study, nanomolar methylene blue concentrations in the brain were sufficient to reduce tau and phospho-tau levels in transgenic mice and to change autophagy markers. We also observed robust reductions in soluble tau levels indicating that methylene blue entered the brain in concentrations sufficient to affect tau levels. We did not observe a concentration of MB in the brain of 500-fold as reported by O'Leary et al who treated with the same paradigm used here - 165 μ M MB in water ad libitum – and reported brain levels of 470 μ M [11]. This could be due to the fact that in our study and in Congdon's study, mice were perfused with saline before dissecting the brain, while they were not in the O'Leary study. Thus some of the methylene blue detected by O'Leary could be in the blood.

A previous study did demonstrate cognitive benefits when conducted at an earlier stage of pathology [11] and methylene blue has potential to enhance proteasome-mediated clearance of amyloid beta [8], enhance autophagy [2], and enhance mitochondrial function [1], indicating that it may still be beneficial in Alzheimer's disease at early points in the illness, but perhaps less so in advanced disease when innumerable tangles already exist.

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Abbreviations

LC	liquid chromatography
MS	mass spectrometry
CA1	Cornu Ammonis area of the hippocampus
TBS	Tris-buffered saline

Highlights

We examined methylene blue treatment in the rTg4510 mouse model of tauopathy.

Methylene blue treatment does not clear existing neurofibrillary tangles in vivo.

Neither PHF1 positive nor Gallyas positive tangles were cleared by the treatment.

Figure 1. Representative micrographs of methylene blue and vehicle treated rTg4510 and wild-type mouse brain

Low magnification micrographs (top) demonstrate marked thinning of the CA1 region of the hippocampus in rTg4510 mice compared to wild-type controls (asterisks) in both vehicle and methylene blue treated groups. Higher magnification images (bottom) demonstrate the presence of PHF1 positive tau inclusions and Gallyas positive neurofibrillary tangles in the cortex and hippocampus of both vehicle and methylene blue treated groups. Scale bars represent 500 μ m (top panels) and 100 μ m (bottom panels).

Figure 2. Stereological quantification of neurofibrillary tangles and neuronal loss in CA1
Stereological estimates of neuron number and PHF1 positive neuron numbers in the CA1 region reveal significant accumulation of neurofibrillary pathology and significant neuronal loss and shrinkage of the CA1 of vehicle treated rTg4510 mice which are not rescued by methylene blue treatment. Asterisks $p < 0.05$ Wilcoxon test.

Figure 3. Stereological quantification of neurofibrillary tangles and neuronal loss in cortex Stereological estimates of neuron number and PHF1 positive neuron numbers in the neocortex reveal significant accumulation of neurofibrillary pathology and significant neuronal loss and shrinkage of the cortex of vehicle treated rTg4510 mice which are not rescued by methylene blue treatment. Asterisks p<0.05 Wilcoxon test.

Figure 4. Biochemical analysis of insoluble tau levels

Sarkosyl extraction and western blots probing for human tau (htau) show no significant reduction in insoluble or soluble tau levels after 6 weeks of methylene blue treatment. Data are presented as means $\pm$ standard error of the mean.