Intravenous Administration of BBB-penetrant, MAPT-silencing, AAV Gene Therapy Provides Broad and Robust CNS Tau Lowering in Tauopathy Mouse Models

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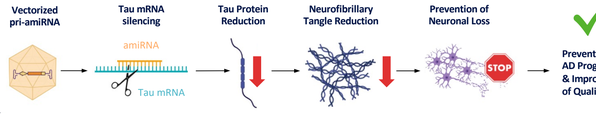
INTRODUCTION

Alzheimer's disease (AD) is a fatal neurodegenerative disease and affects 6 million patients in US and over 50 million patients globally. The patient number is expected to double by 2050, along with trillion dollars of economic burden. Therefore, there is an urgent unmet need to develop a safe and effective drug to treat AD. Progression and spread of tau pathology in the human brain correlates well with cognitive decline in AD. A potential therapeutic approach for AD is to reduce tau protein levels in the central nervous system (CNS). Anti-sense oligonucleotides (ASOs) have been shown to reduce tau expression and tau pathology in preclinical and clinical studies. However, this approach requires repeated administration of the ASO via intra-ventricular or intrathecal administration. Here, we demonstrate that a single, intravenous (IV) administration of a blood-brain barrier (BBB)-penetrant, self-complementary adeno-associated virus (scAAV) gene therapy reduces tau expression in the CNS of tauopathy mouse models expressing human tau.

In silico and *in vitro* screening for microtubule associated protein tau (MAPT) mRNA reduction led to the identification of candidate siRNA sequences that robustly lowered tau mRNA. This was followed by insertion of candidate siRNA sequences into proprietary, primary artificial microRNA (pri-amiRNA) backbones for AAV delivery. The efficacy of one pri-amiRNA candidate, TAU-2023, delivered by a BBB penetrant AAV capsid C9P39, was then thoroughly evaluated in vivo in hTau mice, which express all six isoforms of human MAPT. The results demonstrate a dose-dependent increase in vector genomes and concomitant Tau mRNA and protein knockdown in multiple brain regions of AAV-treated hTau mice, four weeks and eight weeks post-injection. Vectorized TAU-2023 was well-tolerated in hTau mice with no notable test article related changes in body weight or cage-side observations up to eight weeks post dose. Furthermore, vectorized TAU-2023 significantly reduced pathological tau proteins by >95% at eight weeks post-injection in the P301S mouse model, an aggressive tauopathy model, demonstrating robust efficacy. Additionally, small RNA sequencing data indicated favorable efficiency and precision of pri-amiRNA processing.

Results to date indicate that the combination of a potent and well-tolerated, tau-targeting pri-amiRNA and a BBB-penetrant capsid could represent a promising one-time, IV treatment option for AD and other tauopathies.

Figure 1. Biological Hypothesis

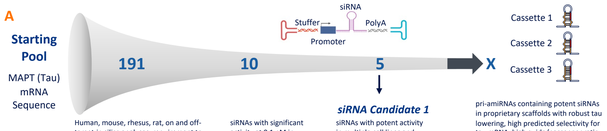


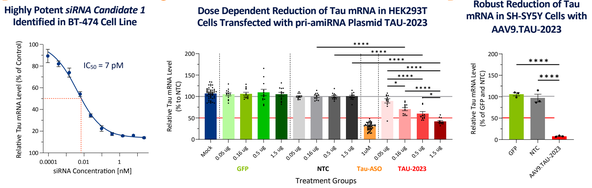
A vectorized pri-amiRNA containing a tau-targeting siRNA sequence is encapsulated by a BBB-penetrant capsid and delivered to the CNS by IV injection. The expressed artificial miRNA will selectively reduce tau mRNA, leading to reductions in tau protein level, tau spread and neurofibrillary tangle accumulation. This should consequently prevent neuronal loss and halt disease progression and cognitive decline, thereby improving quality of life of AD patients.

METHODS

- **Quantitation of Vector Genome Levels by ddPCR:** VG (vector genome) levels were determined following the method described in Wei et al (2021). The number of VG copies in CNS tissue samples was measured with the digital droplet polymerase chain reaction (ddPCR) assay after DNA extraction. Values with ≤5 positive droplets per reaction were defined as below the limit of detection (LOD). The lower limit of quantification (LLOQ) of the assay was 0.01 VG copies/diploid genome.
- **Quantification of MAPT (Tau) mRNA by bDNA or qPCR:**
  - ***In vitro* siRNA Dose Response:** BT474 cells were transfected in a dose-response setup, the highest final siRNA concentration being 24 nM, going down in nine four-fold steps using either LF2000 or RNAiMAX as transfection reagent. The branched DNA (bDNA) assay was used to quantify human Tau, human GAPDH and human AHSA1 (positive control for transfection) mRNAs. The mean ratio of Tau/GAPDH mRNA with all negative control treatments was set to 100% and used for normalization
  - ***In vitro* pri-amiRNA Dose Response:** : HEK293T cells were seeded in 24-well plates, followed by transfection of pri-amiRNA TAU-2023, as well as mock, positive (Tau-ASO) and negative controls. 48 hours of post transfection, cell cultures were harvested for mRNA extraction. Levels of human Tau and human XPNPEP1 mRNA were measured by RT-qPCR. Human Tau mRNA levels were normalized to human XPNPEP1 mRNA levels, and then further normalized to the vehicle control.
  - ***In vitro* AAV9 Transduction:** SH-SY5Y cells were seeded at a density of 25,000 cells/well and transduced with AAV9.TAU-2023, AAV9.GFP, or AAV9.NTC (non-targeting control) at MOI 1E5. 48 hours post transduction, cell cultures were harvested for mRNA extraction. Levels of human Tau and human TBP mRNA were measured by RT-qPCR. Human Tau mRNA levels were normalized to TBP mRNA levels and then further normalized to the GFP and NTC controls.
  - ***In vivo*:** CNS samples were harvested for mRNA extraction. Levels of human Tau and mouse XPNPEP1 (or GAPDH) mRNA in CNS samples were measured by RT-qPCR. Human Tau mRNA levels were normalized to mouse XPNPEP1 (or GAPDH) mRNA levels, and then further normalized to the vehicle control group.
- **Quantitation of Total Tau protein by Immunoassay:** Immunoassay (AlphaLISA (PerkinElmer)) was used to measure total tau protein in soluble fractions isolated from CNS tissue samples based on the manufacturer's protocol.
- **Characterization of the Endogenous miRNA Transcriptome by Small RNAseq:** RNAseq was performed by Fornax Bio. Briefly, purification of total RNA from tissue samples was performed with TRIzol Reagent followed by RNA integrity confirmation with the Bioanalyzer RNA 6000 Nano assay and quantification with the Qubit RNA HS assay. Next, the small RNA-seq library was prepared and quantified with the Qubit dsDNA HS assay, analyzed with the Fragment Analyzer HS NGS assay, and multiplexed according to the requested ratio. NextSeq Sequencing was performed with the 500/550 High-output v2.5 kit (75 cycles) single end 75 bp (SE75) sequencing which yielded ~400 million PF clusters/reads including PhiX (if applied) and followed by bioinformatic analysis.
- **Immunohistochemistry:** In brief, the mouse tissues were fixed in neutral buffered formalin (NBF) for 36-48 hours at room temperature, embedded into paraffin blocks, and sectioned at 5 µm thickness. Immunohistochemistry was performed on the Ventana DISCOVERY ULTRA automated slide preparation system using the OmniMap DAB anti-Rabbit Detection Kit (Ventana, 760-149). Primary antibody, AT100 (Invitrogen, MN1060), was diluted to its working concentration of 1:100 using Rodent Block-M (Biocare Medical, RBM961H). Signal was detected using 3,3'-diaminobenzidine (DAB) and counterstained with Hematoxylin II (Ventana, 790-2208) and Bluing Reagent (Ventana, 760-2037). The slides were scanned using the Leica Aperio GT450 and imaged at 40X magnification.

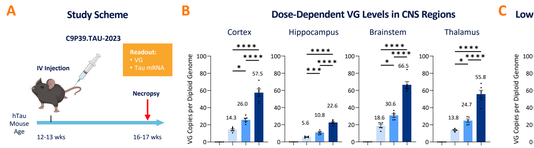
Figure 2. siRNA Screening Funnel and *in vitro* Proof of Concept with Vectorized Pri-amiRNA Candidate TAU-2023

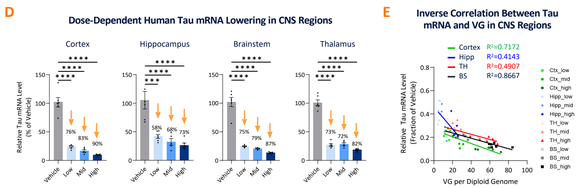




(**A**) Scheme of screening funnel. (**B**) Potent, dose-dependent lowering of Tau mRNA was observed in BT-474 cells following transfection with siRNA candidate 1. A ten-point dose-response experiment was conducted with siRNA candidate 1 at concentrations of 0.000092, 0.00037, 0.0015, 0.0059, 0.0234, 0.0938, 0.375, 1.5, 6, 24 nM, resulting in an impressive IC_{50} of 7 pM and maximal reduction by 90%. Tau mRNA levels were normalized to GAPDH mRNA levels and then further normalized to the mock control. Comparable outcomes were also observed in the HEK293T and LNCap cell lines. (**C**) A dose-dependent decrease in Tau mRNA was observed in HEK293T cells following transfection with a pri-amiRNA containing siRNA candidate 1 (abbreviated as "TAU-2023"). Varying concentrations of TAU-2023, GFP or non-targeting control (NTC; scrambled siRNA) plasmid (0.05, 0.16, 0.5 and 1.5 µg plasmid per well) were transfected using FuGENE HD transfection reagent. Tau antisense oligo-nucleotide (Tau-ASO) that selectively reduces human tau was used as a comparison. Tau mRNA levels were normalized to X-prolyl aminopeptidase 1 (XPNPEP-1) mRNA levels and then further normalized to the NTC. (**D**) Robust 92% reduction of Tau mRNA was observed in SH-SY5Y cells with vectorized TAU-2023 delivered with AAV9 (1E5 MOI). Tau mRNA levels were normalized to TATA-box binding protein (TBP) mRNA levels and then further normalized to the GFP and NTC controls. Panel C-D: Statistical significance was evaluated with a one-way ANOVA and Tukey's multiple comparisons post-hoc test; *, **, ***, and **** indicate p < 0.05, 0.005, 0.0005 and 0.0001, respectively. Data are shown as the group mean ± SEM. C: N≥12 per group, D: N=3 per group.

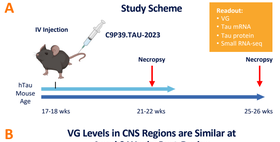
Figure 3. Robust, Dose-Dependent Reduction of Tau mRNA in Key Brain Regions after IV Administration of C9P39.TAU-2023 in hTau Mice

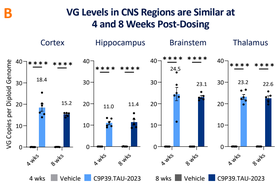


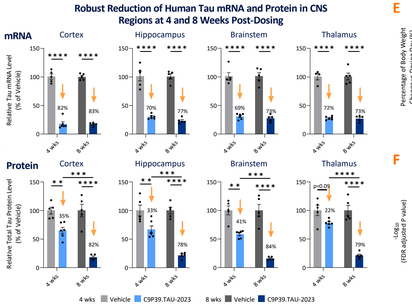


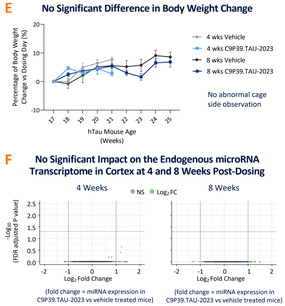
IV administration of C9P39.TAU-2023 in hTau mice resulted in robust and dose-dependent increases in VG with concomitant reductions of Tau mRNA in cortex, hippocampus, brainstem, and thalamus. (**A**) Study design. Twelve- to thirteen-week-old hTau mice were injected intravenously (IV) with vectorized pri-amiRNA TAU-2023 encapsulated in AAV C9P39 capsids (abbreviated as "C9P39.TAU-2023"). Four weeks Later, the cortex (Ctx), hippocampus (Hipp), brainstem (BS), and thalamus (TH) were harvested to quantify vector genome, Tau mRNA and protein levels. (**B**) A dose-dependent increase in vector genome (VG) levels was observed in all evaluated CNS regions. The mean VG levels for each dose group are indicated and ranged from 5.6 to 66.5 VG/diploid cell genome. (**C**) The mean VG levels in the liver of C9P39.TAU-2023-treated mice ranged from 0.42 to 5.48 VG/diploid cell genome. (**D**) A dose-dependent decrease in Tau mRNA was evident in each of the analyzed CNS regions of the C9P39.TAU-2023-treated mice. Tau mRNA levels were normalized to mouse XPNPEP-1 mRNA levels and then further normalized to the vehicle control. Percentage reductions are indicated above each bar. (**E**) There was an inverse correlation between Tau mRNA and VG levels in all the analyzed CNS regions. R^2 value ranged from 0.41 to 0.87. Statistical significance was evaluated with a one-way ANOVA and Tukey's multiple comparisons post-hoc test; *, **, ***, and **** indicate p < 0.05, 0.005, 0.0005 and 0.0001, respectively. Data are shown as the group mean ± SEM. B-E: N=6 per group.

Figure 4. Sustained Tau mRNA and Protein Reduction in Key Brain Regions After IV Administration of C9P39.TAU-2023 in hTau Mice



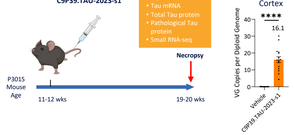


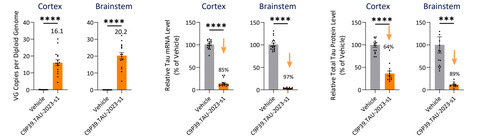


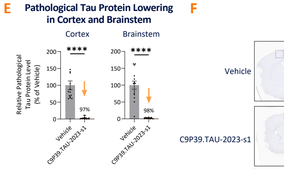


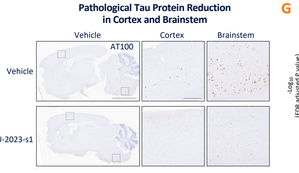
IV administration of C9P39.TAU-2023 in hTau mice resulted in a robust and significant reduction of Tau mRNA and protein in multiple CNS regions at 4- and 8-weeks post injection. (**A**) Study design. Seventeen- to eighteen-week-old hTau mice were injected IV with C9P39.TAU-2023, and the tissues were harvested at 4- or 8-weeks (wks) post injection. DNA and RNA were extracted from the cortex, hippocampus, brainstem, and thalamus samples to quantify vector genome, Tau mRNA and Tau protein levels. Small RNA-seq was also performed on cortex samples. (**B**) Mean VG levels ranging from 11.0 to 24.5 VG/diploid cell genome observed in all evaluated CNS regions. The mean VG levels for each group are indicated. (**C**) A significant decrease in Tau mRNA level was evident in all evaluated CNS regions, at both time points. Tau mRNA levels were normalized to mouse XPNPEP-1 mRNA levels and then further normalized to the vehicle control. (**D**) Robust and significant reductions of total Tau protein level were observed in all evaluated CNS regions, with more reduction at 8 weeks than at 4 weeks post-dosing. Total human tau levels were quantified using AlphaLISA and normalized to the vehicle control. (**E**) There were no significant changes in body weight between vehicle and C9P39.TAU-2023 treated groups at 4- or 8-weeks post-dosing. (**F**) There were no significant difference in the endogenous miRNA expression in the cortex between vehicle and C9P39.TAU-2023 groups at 4- or 8-week time points, based on the miRNA transcriptome profile. The volcano plot shows the −log10 BH-p adjusted value on the y axis and the log2 fold change on the y axis. The y-value indicates statistical significance, or the False Discovery Rate (FDR). The x-value indicates the difference between the dosing groups. Each dot represents one miRNA. Grey dots represent no differences between two groups. Green dots represent the differentiated miRNA expression without statistic significance. The dotted line indicates the p = 0.05, and +/- 2-fold changes on the y and x axis, respectively. No changes achieve significance using DESeq2 analysis. Panels B-D: statistical significance was evaluated with a two-way ANOVA and Sidak's multiple comparisons post-hoc test; *, **, ***, and **** indicate p < 0.05, 0.005, 0.0005 and 0.0001, respectively. Panel E: two-way RM ANOVA were performed with Sidak's multiple comparisons post-hoc test. Data are shown as the group mean ± SEM. B-D: N=5-6 per group. E: N=3. Percentage reductions are indicated above each bar.

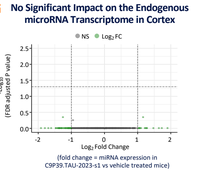
Figure 5. Robust Reduction of Tau Pathology in Key Brain Regions after IV Administration of C9P39.TAU-2023-s1 in P301S Mice











IV administration of C9P39.TAU-2023-s1 in P301S mice resulted in robust and significant reductions of pathological Tau protein in cortex and brainstem. (**A**) Study design. Eleven- to twelve-week-old P301S-mice were injected IV with C9P39.TAU-2023 driven by a neuronal promoter (abbreviated as "C9P39.TAU-2023-s1"). The cortex and brainstem samples were harvested 8-weeks post dose to quantify vector genome, Tau mRNA, total Tau protein and pathological Tau protein levels. Small RNA-seq was also performed on cortex samples. (**B**) Mean VG levels were 16.1 and 20.2 VG/diploid cell genome in the cortex and brainstem of C9P39.TAU-2023-s1-treated P301S mice, respectively. (**C**) A significant decrease in Tau mRNA level was evident in all evaluated CNS regions. Tau mRNA levels were normalized to mouse XPNPEP-1 mRNA levels and then further normalized to the vehicle control. (**D**) Robust, significant reduction of total tau protein levels was observed in cortex and brainstem of P301S mice dosed with C9P39.TAU-2023-s1. Total human tau levels were quantified using AlphaLISA and normalized to the vehicle control. (**E**) Robust, significant reduction of pathological tau protein levels was observed in cortex and brainstem of P301S mice dosed with C9P39.TAU-2023-s1. Pathological human tau levels were quantified using AT8 ELISA and normalized to the vehicle control. (**F**) Histology images showing substantial reduction of pathological tau protein immunostained by AT100 in P301S mice dosed with C9P39.TAU-2023-s1. Magnified images highlight cortex and brainstem regions. Scale bars for overview and magnified images are 3 mm and 0.4 mm, respectively. Same scale for both groups. (**G**) There was no significant difference in the endogenous miRNA expression in the cortex between vehicle and C9P39.TAU-2023-s1 treatment groups, based on the miRNA transcriptome profile. The volcano plot shows the −log10 BH-p adjusted value on the y axis and the log2 Fold change on the y axis. The y-value indicates statistical significance, or the False Discovery Rate. The x-value indicates the difference between the dosing groups. Each dot represents one miRNA. Grey dots represent no differences between two groups. Green dots represent the differentiated miRNA expression without statistic significance. The dotted line indicates the p = 0.05, and +/- 2-fold changes on the y and x axis, respectively. No changes achieve significance using DESeq2 analysis. Panels B-E: statistical significance was determined with unpaired two-tailed t-test, *, **, ***, and **** indicate p < 0.05, 0.005, 0.0005 and 0.0001, respectively. Data are shown as the group mean ± SEM. B-E: N=15-18 per group. F-G: N=3. Percentage reductions are indicated above each bar.

CONCLUSIONS

- TAU-2023, a vectorized tau-targeting pri-amiRNA elicits dose-dependent increase in vector genomes and concomitant tau mRNA knockdown in key brain regions of treated hTau mice, four weeks post-IV administration.
  - Significant tau mRNA reduction were observed, ranging from 58% to 90% relative to the vehicle-treated hTau mice.
- TAU-2023 significantly reduced total tau protein from 22% to 41% at four weeks post-injection, and 78% to 84% at eight weeks post-injection, in multiple brain regions of hTau mice, relative to baseline levels.
- TAU-2023 showed over 95% reduction of pathological tau protein in the cortex and brainstem of P301S mice, eight weeks post-injection.
- Small RNA-seq analysis in the cortex of both hTau and P301S mice treated with TAU-2023 indicates no significant impact on the endogenous microRNA transcriptome at doses that result in significant reduction of cortical tau protein.
- In summary, these results show that the combination of a potent and well-tolerated Tau targeting pri-amiRNA and a BBB-penetrant capsid represents a promising one-time, IV treatment option for AD and other tauopathies.