An anti-neuroinflammatory that targets dysregulated glia enhances the efficacy of CNS-directed gene therapy in murine infantile neuronal ceroid lipofuscinosis

Shannon L. Macauley  
*Washington University School of Medicine in St. Louis*

Andrew M. S. Wong  
*King's College London - Institute of Psychiatry*

Charles Shyng  
*Washington University School of Medicine in St. Louis*

David P. Augner  
*King's College London - Institute of Psychiatry*

Joshua T. Dearborn  
*Washington University School of Medicine in St. Louis*

See next page for additional authors

Follow this and additional works at: [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)

Please let us know how this document benefits you.

**Recommended Citation**
Macauley, Shannon L.; Wong, Andrew M. S.; Shyng, Charles; Augner, David P.; Dearborn, Joshua T.; Pearse, Yewande; Roberts, Marie S.; Fowler, Stephen C.; Cooper, Jonathan D.; Watterson, D. Martin; and Sands, Mark S., "An anti-neuroinflammatory that targets dysregulated glia enhances the efficacy of CNS-directed gene therapy in murine infantile neuronal ceroid lipofuscinosis." The Journal of Neuroscience. 34, 39. 13077-13082. (2014).  
[https://digitalcommons.wustl.edu/open_access_pubs/3362](https://digitalcommons.wustl.edu/open_access_pubs/3362)

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.
Authors
Shannon L. Macauley, Andrew M. S. Wong, Charles Shyng, David P. Augner, Joshua T. Dearborn, Yewande Pearse, Marie S. Roberts, Stephen C. Fowler, Jonathan D. Cooper, D. Martin Watterson, and Mark S. Sands
Infantile neuronal ceroid lipofuscinosis (INCL) is an inherited neurodegenerative lysosomal storage disease (LSD) caused by a deficiency in palmitoyl protein thioesterase-1 (PPT1). Studies in Ppt1−/− mice demonstrate that glial activation is central to the pathogenesis of INCL. Astrocyte activation precedes neuronal loss, while cytokine upregulation associated with microglial reactivity occurs before and concurrent with neurodegeneration. Therefore, we hypothesized that cytokine cascades associated with neuroinflammation are important therapeutic targets for the treatment of INCL. MW01–2-151SRM (MW151) is a blood–brain barrier penetrant, small-molecule anti-neuroinflammatory that attenuates glial cytokine upregulation in models of neuroinflammation such as traumatic brain injury, Alzheimer’s disease, and kainic acid toxicity. Thus, we used MW151, alone and in combination with CNS-directed, AAV-mediated gene therapy, as a possible treatment for INCL. MW151 alone decreased seizure susceptibility. When combined with AAV-mediated gene therapy, treated INCL mice had increased life spans, improved motor performance, and eradication of seizures. Combination-treated INCL mice also had decreased brain atrophy, astrocytosis, and microglial activation, as well as intermediary effects on cytokine upregulation. These data suggest that MW151 can attenuate seizure susceptibility but is most effective when used in conjunction with a therapy that targets the primary genetic defect.

Key words: Batten disease; lysosomal storage disease; neurodegeneration; neuroinflammation; neuronal ceroid lipofuscinosis
based on body weight or volume. Therefore, dosing of AAV-mediated enzyme replacement to the CNS is a challenge and not readily amenable to the optimization routinely accomplished with small-molecule therapeutics. However, promising replacement therapies can be combined with various complementary treatments based on dosing considerations. Because the enzyme deficiency in the Ppt1−/− mice is accompanied by neuroinflammation and increased cytokine production, a rational approach would be to test the potential of an experimental therapeutic such as MW151 as an adjunct therapy. MW151 is a glial-specific, small molecule whose pharmacological mechanism of action is attenuation of injurious upregulation of pro-inflammatory cytokines, which are associated with brain injury, progressive neurodegeneration, or increased pathology susceptibility due to CNS innate immunity priming (Hu et al., 2007; Somera-Molina et al., 2007; Karpus et al., 2008; Chrzaszcz et al., 2010; Bachstetter et al., 2012).

We hypothesized that simultaneous treatment with the small-molecule drug MW151 would improve the outcome of CNS-directed, AAV2/5-mediated gene therapy. In fact, this cotreatment resulted in increased life span, improved motor function, elimination of seizures, and decreased glial activation and cytokine production. The outcomes demonstrate the potential utility of using drugs that attenuate pro-inflammatory cytokine production as a cotherapy for gene-mediated replacement therapy for INCL.

Materials and Methods

**Ppt1−/− and wild-type mice.** Congenic wild-type (+/+) or Ppt1−/− (−/−) mice on the C57BL/6 background were generated at Washington University School of Medicine. Genotype was determined by PCR-based assay (Gupta et al., 2001). Male and female Ppt1−/− mice and normal littermates were used. All procedures were performed in accordance with an approved IACUC protocol from Washington University School of Medicine.

**Life span.** Longevity was assessed in treated Ppt1−/− mice and sham-treated controls (n = 10–15 mice/group). End of life was designated by death or a predetermined moribund condition. Kaplan–Meier analysis was used to measure cumulative survival and establish significant differences in life span (p < 0.05).

**Recombinant AAV production.** The rAAV2/5-PPT1 vector used in these studies was generated at the University of Florida Vector Core Laboratory as previously described (Griffey et al., 2004). Briefly, the vector contained the cytomegalovirus enhancer, chicken β-actin promoter; first intron from the chicken β-actin gene; human PPT1 cDNA; rabbit β-globin polyadenylation signal; and flanking inverted terminal repeats from AAV2.

**Intracranial injections.** On P1, the viral vector was injected intracranially using a Hamilton syringe and 30 gauge needle. Two microliters of virus (1 × 10^12 vg/ml) was bilaterally injected into the anterior cortex (1 mm rostral to bregma, 2 mm mediolateral of midline, and 2 mm ventral to the surface of the skull), hippocampus/thalamus (3.5 mm rostral to bregma, 2 mm mediolateral of midline, and 2 mm ventral to the surface of the skull), and cerebellum (1 mm rostral to lambda, 1 mm mediolateral of midline, and 2 mm ventral to the surface of the skull).

**MW151 injections.** A 1 × MW151 solution was made daily in sterile 0.9% saline, filter sterilized, and immediately injected. Ppt1-deficient mice received daily intraperitoneal injections of MW151 at a dose of 2.5 mg/kg. Dosing began at 28 d of age and continued for the remainder of their life. Additionally, separate cohorts of Ppt1−/− and WT mice received daily injections of saline and served as controls.

**Treatment groups.** Five treatment groups were used in this study: (1) WT; WT mice receiving daily saline injections. (2) Ppt1−/−; Ppt1−/− mice receiving daily saline injections. (3) MW151; Ppt1−/− mice receiving daily injections of MW151, (4) AAV; Ppt1−/− mice receiving intracranial injections of AAV2/5-PPT1, and (5) AAV + MW151: Ppt1−/− mice receiving both intracranial injections of AAV2/5-PPT1 and daily MW151 injections. Each group contained a total of 20 randomly assigned, mixed-sex mice.

**Rotarod testing.** Treated and sham-treated mice (n = 10–13 mice/group) were tested on the constant-speed rotarod beginning at 4 months of age, each month thereafter as previously described (Macauley et al., 2012). Statistical significance was determined using one-way ANOVAs at each time point followed by post hoc tests.

**Seizure monitoring via force plate actometer.** A force plate actometer was teamed with simultaneous video monitoring to assess seizure activity within treated and untreated mice at 7.5 months of age. Briefly, a custom-made actometer was generated as previously described (Reddy et al., 2011). Simultaneous video monitoring and force plate actometry were gathered for 8 consecutive hours. Detection of a seizure with the actometer was confirmed by visually observing a seizure (“popcorn” seizure followed by freezing) on the video recording during the same time frame. Significant differences in seizure activity between untreated Ppt1−/− mice and treated animals were determined using a χ² test.

**PPT1 activity.** At 7.5 months of age, treated and sham-treated mice (n = 3–6) were killed via lethal injection, the brains bisected sagittally, and the left hemisphere flash frozen in liquid nitrogen. PPT1 assays were performed on brains as previously described (Macauley et al., 2012). The values were normalized to total protein measured using a Coomassie dye-binding assay (Bio-Rad Laboratories). One-way ANOVA and Tukey’s multiple-comparison tests were used to determine significance.

**Brain atrophy.** At 7.5 months, treated and sham-treated mice (n = 3–9) were killed and the brains dissected by a researcher blinded to both age and genotype as previously described (Macauley et al., 2011). Each brain was weighed and differences in brain weights were analyzed with one-way ANOVAs followed by Bonferroni correction post hoc tests.

**Brain processing, Nissl staining, and regional volume measurements.** At 7.5 months of age, brains from treated and sham-treated mice (n = 4 mice/group) were removed and fixed for 48 h in 4% paraformaldehyde in PBS followed by cryoprotection in a 30% sucrose solution. One hemisphere of each forebrain and the cerebellum were sectioned on a freezing microtome at 40 μm. For Nissl staining, sections were mounted onto coated SuperFrost microscope slides, allowed to air dry overnight, and stained with a 0.05% cresyl fast violet solution. Unbiased estimates of the volumes of cortex, hippocampus, striatum, and thalamus were obtained via the Cavalieri method as previously described. Statistical differences were determined using one-way ANOVAs followed by Bonferroni correction post hoc tests.

**Immunohistochemistry for glial markers.** Immunostaining and quantification for GFAP and CD68 were performed in regions of interest as previously reported (Macauley et al., 2012). One-way ANOVAs with Bonferroni correction post hoc tests were used to determine statistical significance.

**Analysis of cytokine profiles.** To quantify the concentration of chemokines and cytokines within the brain (n = 3–4 mice per group), a 14-biomarker Multi-Analyte Profile was generated for 7.5-month-old treated and sham-treated mice by Rules Based Medicine using standard Luminex Technology (Luminex) for 14 separate analytes as previously described (Macauley et al., 2011). One-way ANOVA followed by Bonferroni post hoc tests was used to determine significant changes.

**Results**

**Life span.** One measure of therapeutic efficacy is longevity. Ppt1−/− mice die at ~34.9 weeks compared with normal littermates (Fig. 1A). Daily injections of MW151 alone did not significantly increase life span compared with sham-treated Ppt1−/− mice (35.4 vs 34.9 weeks, respectively). However, treatment with AAV alone or AAV + MW151 injections led to significant increases in median survival. Moreover, AAV + MW151 treatment resulted in a significant increase in life span compared with AAV alone (47 vs 45.4 weeks, respectively).
Constant speed rotarod
We investigated the combined effect of AAV-mediated gene therapy and daily MW151 injections on motor deficits in the Ppt1−/− mouse (Fig. 1B). At 4 months, all treated and sham-treated mice mastered the constant speed paradigm and performed similar to WT. Beginning at 6 months, motor function in sham-treated and treated Ppt1−/− mice deteriorated when compared with AAV-only, AAV + MW151, and WT mice. The performance of Ppt1−/− or MW151-only mice continued to decline until their death at 7 and 8 months, respectively. In contrast, mice treated with AAV only or AAV + MW151 performed comparably to normal littermates through 7 months. At 8 months, the AAV + MW151 group performed significantly (p < 0.05) better than the AAV-only group. The performance of AAV-only and AAV + MW151-treated mice continued to deteriorate at 9 months, culminating in a nadir in performance at 10 months.

Seizure activity
Using a force plate actometer combined with video monitoring, seizure activity was examined in treated and sham-treated mice (Fig. 1C). At 7.5 months, 43.8% of Ppt1−/− mice had at least one spontaneous seizure during the 8 h monitoring session. In contrast, no spontaneous seizure activity was observed in WT mice. Ppt1-deficient mice treated with MW151 alone displayed decreased seizure activity, where only 1 of 6 mice tested had seizures. Treatment with AAV only or AAV + MW151 eradicated the seizure phenotype.

PPT1 activity
PPT1 activity in the normal brain is ∼393.2 nmol/mg/h (Fig. 1D). In contrast, PPT1 activity was significantly decreased in the brains of untreated Ppt1−/− and MW151-treated mice (10.8 and 8.5 nmol/mg/h, respectively). Following AAV-only or AAV + MW151 treatment, there is a significant increase in PPT1 activity to 504.1 and 640.3 nmol/mg/h, respectively.

Overall brain atrophy
Brain weight measurements were used as a simple means to determine overall brain atrophy in treated and untreated mice (Fig. 2A). In the Ppt1−/− brain, there was a significant decrease in brain weight, by ∼20%, at 7.5 months compared with WT mice (p < 0.001). Treatment with MW151 alone had no effect on overall brain atrophy. In contrast, the brain weights from AAV-only and AAV + MW151 groups were significantly increased compared with untreated Ppt1−/− controls and were comparable to WT.

Regional atrophy and neuronal loss
There was a significant reduction in regional volume within the cortex, hippocampus, thalamus, and cerebellum of Ppt1−/− mice compared with WT (Fig. 2B–E). Daily injections of MW151 did not alter regional atrophy in any of the regions assayed. In contrast, treatments including intracranial delivery of AAV or AAV + MW151 resulted in significant increases in cortical and thalamic volume. Interestingly, the combination of AAV + MW151 significantly increased hippocampal volume (p < 0.05) while treatment with AAV alone did not. There was a significant decrease in calbindin-positive Purkinje cells within the cerebellum of Ppt1−/− mice. Treatment with MW151 alone did not decrease neuronal loss within the cerebellum. Although treatment with AAV or AAV + MW151 did not significantly increase Purkinje cell number, there appeared to be an increase in Purkinje cell number in the AAV + MW151 group that did not reach significance.
Regional neuroinflammation

Astrocyte activation (increased GFAP expression; Fig. 3A, B) and microglial reactivity (increased CD68 expression; Fig. 3C, D) are key components of INCL neuropathology. There is a significant increase in astrocyte activation at 7.5 months in the cortex, thalamus, hippocampus, and cerebellum of Ppt1−/− mice compared with WT controls (Fig. 3B). Treatment with MW151 alone did not decrease astrocyte activation in any of the brain regions assayed. However, treatment with AAV or AAV + MW151 significantly decreased astrocyte activation within cortex, thalamus, and hippocampus. Interestingly, there was no effect observed with either treatment in the cerebellum.

Profound microglial reactivity is observed within the Ppt1−/− cortex, thalamus, hippocampus, and cerebellum at 7.5 months (Fig. 3C, D). Following treatment with MW151 alone, there appeared to be a decrease in CD68 staining within the cortex, although this was not significant. Treatment with AAV and AAV + MW151 resulted in a significant decrease (p < 0.01) in microglial activation within the cortex and hippocampus. Similarly, CD68 immunostaining appeared to decrease in the thalamus of mice treated with MW151 alone, AAV only, or AAV + MW151, but this effect did not reach significance. Also, combination therapy with AAV + MW151 resulted in a significant decrease in microglial activation in Ppt1−/− cerebellum (p < 0.05), while AAV alone had little effect.

Cytokine production

Previous reports describe a significant increase in cytokine expression in Ppt1−/− brains as disease progresses (Qiao et al., 2007; Kielar et al., 2009; Macauley et al., 2011). Therefore, we sought to measure the levels of various pro-inflammatory markers such as IFN-γ and TNF-α (Fig. 4A); lymphocyte/monocyte mediators, such as Oncostatin M and IP-10 (Fig. 4B); and monocyte mediators, such as MIP-1β, MIP-2, and MCP-1 (Fig. 4C). At 7.5 months, pro-inflammatory cytokines as well as monocyte and lymphocyte mediators are elevated in Ppt1−/− mice compared with littermate controls. Although no significant effects on cytokine levels were observed with any
Figure 4. Therapeutic intervention decreases cytokine production in the Ppt1+/− brains. Luminescence assays were performed for (A) pro-inflammatory markers, (B) lymphocyte/monocyte activators, and (C) monocyte activators. Elevations in the cytokines, IFN-γ, TNF-α, MIP-1β, MIP-2, MCP-1, Oncostatin M, and IP-10, were observed in Ppt1+/− mice. Treatment with AAV only and AAV + MW151 produced a trend toward a decrease in IFN-γ, TNF-α, MIP-1β, MIP-2, MCP-1, Oncostatin M, and IP-10, although the change was not statistically significant. Error bars represent ± SEM.

Discussion

Previous studies in the Ppt1−/− mice demonstrated that neuroinflammation is central to the pathogenesis of INCL (Kielar et al., 2007; Macauley et al., 2009, 2011). Widespread astrocytosis and microglial activation accompany this frank neurodegenerative disorder. Before the onset of neurodegeneration, astrocyte activation and microglial activation accompany this frank neurodegenerative disorder. Concurrent with these processes, there is an influx of monocytes and leukocytes into the CNS. Recent studies also demonstrated that inactivation of the adaptive immune system in Ppt1−/− mice spared axonal damage and neuronal loss (Groh et al., 2013). Therefore, increased expression of cytokines within the CNS and the infiltration of peripheral immune cells are closely associated with the neuronal pathology and functional decline. Given the evidence implicating pro-inflammatory cytokine-mediated neuroinflammation in Ppt1+/− mice, we investigated the therapeutic value of MW151, a small molecule capable of mitigating glial activation and cytokine production, as a cotherapeutic approach with CNS-directed, AAV-mediated gene therapy in the mouse model of INCL (Macauley et al., 2012; Roberts et al., 2012).

AAV-mediated gene therapy alone resulted in significant biochemical, histological, and behavioral improvements in PPT1-deficient mice. The unexpected main positive finding of MW151 treatment alone was the significant decrease in seizure incidence in the Ppt1−/− mice compared with untreated controls. This is an important finding given the severity of the seizure phenotype in INCL. In addition, this suggests that seizures associated with INCL might be related to the neuroinflammatory response associated with neuronal dysfunction. However, MW151 did not decrease histopathological markers of disease. This is not entirely surprising since MW151 does not target the primary defect in Ppt1−/− mice, an enzyme deficiency which results in accumulation of undegraded material. Long-term treatment (every day for nearly 1 year) with MW151 at 2.5 mg/kg was well tolerated but might not be sufficient given the severity of disease progression. It is possible that chronic treatment with even higher doses would be well tolerated and perhaps result in greater benefit alone or in combination with gene therapy. A dose-ranging study showed that chronic administration of MW151 at doses >100-fold higher than those reported here were well tolerated and resulted in little or no toxicity (D.M. Watterson, personal communication).

In previous studies (Macauley et al., 2011; Hu et al., 2012; Roberts et al., 2012) that target the primary defect such as CNS-directed gene therapy or ERT, clinical improvement was modest but improved with use of adjunct therapies. This is likely due to the aggressive and complex nature of this disease. INCL is a chronic disease with a rapid clinical course, affecting the brain in its entirety. This poses a unique set of challenges for replacement therapy. Only addressing the enzymatic deficit in INCL patients will likely yield partial results. However, when used in combination with drugs such as MW151, there is the additional benefit of targeting a secondary pathogenic mechanism in conjunction with the appeal of less invasive dosing. Beyond INCL, this has implications for treating other forms of Batten disease, such as late-infantile and juvenile NCL, given that glial activation and seizure deficits are prominent features of these diseases as well (Oswald et al., 2005; Chang et al., 2008; Chen et al., 2009; Weimer et al., 2009). The concept of combining replacement therapy with small-molecule cotherapy is also gaining acceptance in other areas of neurotherapeutics (Glover et al., 2012). Overall, the results reported here demonstrate the potential of using AAV-mediated enzyme replacement therapy combined with mechanism-based, small-molecule drug treatment for INCL.
References


Macauley et al. • Anti-Inflammatory Therapy for INCL