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An Anti-Neuroinflammatory That Targets Dysregulated Glia Enhances the Efficacy of CNS-Directed Gene Therapy in Murine Infantile Neuronal Ceroid Lipofuscinosis

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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

Introduction

Infantile neuronal ceroid lipofuscinosis (INCL, or CLN1 disease) is a rare, autosomal recessive lysosomal storage disease that largely affects the CNS beginning in infancy or early childhood (Vesa et al., 1995; Hofmann et al., 1999). INCL is caused by mutations in the CLN1 gene leading to a deficiency in palmitoyl protein thioesterase-1 (PPT1). Typically, patients are asymptomatic at birth and development proceeds normally. However, by 2 years of age, INCL patients suffer from blindness, seizures, cognitive impairment, and motor dysfunction, ultimately leading to death by 4–6 years of age.

Previous studies demonstrate that PPT1-deficient (Ppt1−/−) mice mimic the clinical features of INCL, such as cognitive, motor, and visual deficits, as well as spontaneous seizures. Interestingly, focal areas of astrocyte activation were the first pathological feature observed in the Ppt1−/− brain (Kielar et al., 2007; Macauley et al., 2009, 2011; Groh et al., 2013). Coincident with the onset of astrocyte activation was a significant increase in pro-inflammatory cytokines, microglial activation, and leukocyte recruitment into the brain. Specific brain regions affected by early astrocyte activation were the same regions prone to neurodegeneration and brain atrophy.

Preclinical intervention studies for INCL reveal the promise for enzyme replacement therapy (ERT) via CNS-directed, AAV-mediated gene therapy (Griffey et al., 2004, 2006; Macauley et al., 2012; Roberts et al., 2012). However, this approach has proven to be only partially efficacious given the complexity of clinical features associated with INCL. Therefore, isolating secondary disease mechanisms that are responsive to therapeutic intervention is necessary for improved dosing and efficacy. Dosing includes a therapeutic time window, based on pathophysiology progression; frequency of administration, based on how the body reacts to the treatment; as well as the amount of drug administered,
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; Bachsteller 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$^{-/-}$ 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 $\beta$-actin promoter; first intron from the chicken $\beta$-actin gene; human PPT1 cDNA; rabbit $\beta$-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 $\times$ 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 $\times$ 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.

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 AAV-only group. The performance of AAV-only and AAV + MW151 treatment, there is a significant increase in PPT1 activity from 504.1 and 640.3 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.

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/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.

Figure 1. A, Increased median life span was observed with treatment of AAV only or AAV + MW151 in Ppt1/−/− mice. No improvement in life span was seen with MW151 alone. B, AAV alone significantly improved performance on the constant-speed rotarod test. AAV + MW151 provided a further increase in motor performance at 8 months of age. C, Treatment with MW151 alone, AAV alone, or the combination, decreased seizure frequency in Ppt1/−/− mice. Untreated Ppt1/−/− mice (n = 16) had a total of 11 seizures during the monitoring period, while no WT mice (n = 20) had seizures. Ppt1/−/− mice treated with MW151 only (n = 6) had a single seizure during the same monitoring period. Moreover, AAV only (n = 24), or AAV in combination with MW151 (n = 6), eradicated this seizure phenotype. D, Compared with Ppt1/−/− brains, there is a significant increase in PPT1 activity following treatment with AAV only or AAV + MW151. Daily injections of MW151 did not increase PPT1 activity (*p < 0.05; **p < 0.01; ***p < 0.001). Error bars represent ± SEM.

Figure 2. A, Brain weight, a marker of CNS atrophy, increased in Ppt1/−/− mice following therapeutic intervention with AAV only or AAV + MW151 compared with untreated Ppt1/−/− mice. B, Cortical and thalamic volumes increased significantly following AAV-only or AAV + MW151 therapy. D, Hippocampal volume was significantly increased following AAV + MW151 therapy. E, There was no significant increase in cerebellar volume after therapeutic intervention (*p < 0.05; **p < 0.01; ***p < 0.001). Error bars represent ± SEM.
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<sup>−/−</sup> mice compared with WT controls (Fig. 3B). Treatment with MW151 alone had little effect on GFAP immunoreactivity. C, There was a significant decrease in microglial activation in the cortex and hippocampus of Ppt1<sup>−/−</sup> mice following AAV or AAV + MW151 therapy. The combination of AAV + MW151 also significantly decreased CD68 staining in the cerebellum. There was a trend toward a decrease in CD68 staining in the MW151-only group in the cortex and thalamus, but this did not reach significance (*p < 0.05; **p < 0.01; ***p < 0.001). Error bars represent ± SEM.

Cytokine production

Previous reports describe a significant increase in cytokine expression in Ppt1<sup>−/−</sup> brains as disease progresses (Qiao et al., 2007; Kiellor 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<sup>−/−</sup> cerebellum (p < 0.05), while AAV alone had little effect.
treatment, all treatments appeared to decrease levels within the brain. Specifically, AAV-only and AAV + MW151 groups appeared to have more robust effects on all cytokine assays. However, the MW151-alone appeared to decrease several monocyte (MIP-2 and MCP-1) and monocyte/leukocyte (Onco
costatin M) activators, and (C) monocyte activators. Elevations in the cytokines, IFN-γ, TNF-α, MIP-1B, 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-1B, MIP-2, MCP-1, Oncostatin M, and IP-10, although the change was not statistically significant. Error bars represent ± SEM.

Figure 4. Therapeutic intervention decreases cytokine production in the Ppt1−/− brains. Luminex 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-1B, 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-1B, 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 neuro-
inflammation 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 occurs in specific brain regions that ultimately suffer neuronal loss and brain atrophy. In addition to astrocytosis, microglial activation occurs before and during neuronal cell loss. Concurrent with glial activation, the Ppt1−/− brain displays elevated levels of chemokines and cytokines, which are responsible for the recruitment 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


