Plastin-3 extends survival and reduces severity in mouse models of spinal muscular atrophy

Kevin A. Kaifer,1,2 Eric Villalón,2,3 Erkan Y. Osman,2,3 Jacqueline J. Glascock,1 Laura L. Arnold,4 D.D.W. Cornelison,2,4 and Christian L. Lorson1,2,3

1Molecular Pathogeneses and Therapeutics Program, 2Bond Life Sciences Center, 3Department of Veterinary Pathobiology, College of Veterinary Medicine, 4Division of Biological Sciences, University of Missouri, Columbia, Missouri, USA.

Introduction

Spinal muscular atrophy (SMA) is a leading genetic cause of infantile death and is caused by the loss of survival motor neuron-1 (SMN1). Importantly, a nearly identical gene is present called SMN2; however, the majority of SMN2-derived transcripts are alternatively spliced and encode a truncated, dysfunctional protein. Recently, several compounds designed to increase SMN protein have entered clinical trials, including antisense oligonucleotides (ASOs), traditional small molecules, and gene therapy. Expanding beyond SMN-centric therapeutics is important, as it is likely that the breadth of the patient spectrum and the inherent complexity of the disease will be difficult to address with a single therapeutic strategy. Several SMN-independent pathways that could impinge upon the SMA phenotype have been examined with varied success. To identify disease-modifying pathways that could serve as stand-alone therapeutic targets or could be used in combination with an SMN-inducing compound, we investigated adeno-associated virus–mediated (AAV-mediated) gene therapy using plastin-3 (PLS3). Here, we report that AAV9-PLS3 extends survival in an intermediate model of SMA mice as well as in a pharmacologically induced model of SMA using a splice-switching ASO that increases SMN production. PLS3 coadministration improves the phenotype beyond the ASO, demonstrating the potential utility of combinatorial therapeutics in SMA that target SMN-independent and SMN-dependent pathways.
this notion, two compounds have entered clinical trials for SMA that are mechanistically distinct from the potential therapeutics that increase SMN: a neuroprotectant being developed by Roche (Olesoxime) and a skeletal muscle activator being developed by Cytokinetics (CK-2127107) (10, 11). Going forward, novel SMN-independent targets for drug development will likely play an important role in treating SMA, and genetic modifiers of SMA are a likely source for SMN-independent drug targets.

Excluding SMN2, the most promising genetic modifier target is plastin-3 (PLS3), a calcium-dependent actin-bundling protein. PLS3 upregulation correlates with reduced severity in SMA-discordant siblings, and transient expression of PLS3 in Smn-deficient zebrafish can rescue motor neuron pathology (12–15). To investigate the therapeutic potential of a genetic modifier of the SMA phenotype, we utilized adenovirus-associated virus serotype 9 (AAV9) to deliver the PLS3 cDNA. AAV9 exhibits a broad tropism for tissues within the CNS and within peripheral tissues (16–19). Importantly, AAV9-mediated delivery enters disease-relevant cells and is able to quickly express the transgene, as evidenced by the significant rescue of the SMA phenotype following AAV9-SMN delivery (18, 20–23). Here, we show that AAV9-mediated delivery of PLS3 significantly extended survival in two important disease models of SMA: administration of PLS3 as a stand-alone strategy in an model of intermediate disease (SmnΔ7+/– mice) and coadministration of PLS3 and an SMN-increasing antisense oligonucleotide (ASO) in a model of severe disease (mSmnΔ7–/–, hSMN2+/+; hSMNΔ7–/+ mice, referred to as SMNΔ7 mice). In addition, we demonstrate that viral delivery of PLS3 can improve motor function, reduce skeletal muscle atrophy, and improve neuromuscular junction (NMJ) integrity and maturation, validating PLS3 as a true modifier of SMA and supporting the idea of combinatorial therapy as a viable avenue for the treatment of SMA.

Results

The initial objective was to determine if AAV9-PLS3 could improve the SMA phenotype in SMA model of severe disease, SMAΔ7 mice. 1 × 1011 vector genomes of self-complimentary AAV9–PLS3 (scAAV9–PLS3; scAAV9 is herein referred to as AAVP) did not significantly increase the survival of severe SMNΔ7 SMA mice nor was disease progression altered (Figure 1A). In a separate report, a similar severe SMA model did not show a significant life span extension when PLS3 was expressed as a transgene, consistent with the current results and demonstrating that SMN-independent strategies are likely better suited for less severe disease contexts (12). In contrast, AAV9-PLS3 treatment of an intermediate SMA model, SmnΔ7+/– mice, with 1 × 1011 vector genomes resulted in a significant extension of survival (~71%) compared with untreated cohorts (Figure 1B). When the dose was increased to 3 × 1011 vector genomes, AAV9-PLS3 did not further extend survival but resulted in a similar extension of survival, demonstrating that these levels did not result in overt toxicity issues. While life span was extended, an increase in weight was not observed in AAV9-PLS3–treated animals (Figure 1C).

To validate expression of the AAV9-PLS3 vector in disease-relevant tissue, a Western blot was performed using tissue extract generated from spinal cord samples from treated and untreated mice (Figure 1D). PLS3 levels were elevated compared with baseline levels following a single injection via the facial vein of 1 × 1011 vector genomes of the AAV9-PLS3 vector. Widespread distribution from a single peripheral injection has been reported extensively in SMA and related fields (16–19); therefore, achieving spinal cord expression from a peripheral injection was expected. Collectively, these results provide proof-of-concept evidence that increasing PLS3 levels could be an alternative approach for reducing the severity in milder forms of SMA.

Mouse models of severe SMA are characterized by significant multisystem dysfunction (24) that likely prevents PLS3 or other SMN-independent modifiers from significantly affecting disease severity. However, increasing SMN levels, even slightly, might be sufficient to restore the function of various organ systems enough to reveal modifier-dependent effects. To examine the potential application of PLS3 with a SMN-increasing compound as a means to explore the potential development of combinatorial therapeutics, AAV9-PLS3 and a previously developed splice-switching ASO were coadministered to SMNΔ7 mice. The selected ASO, referred to as MOE1v11, targets a potent intronic splice silencer called element 1 and is located proximal to exon 7 (25, 26). To examine the utility of combinatorial therapeutics in SMA, SMN levels were incrementally increased by establishing a dose response with MOE1v11, thereby identifying functional, yet suboptimal ASO doses. Based upon our previous work, two initial MOE1v11 doses were examined to establish a suboptimal baseline: a 5-μl injection consisting of either 1 or 2 nmol of MOE1v11 (25). The MOE1v11 ASO was administered at P1 via an intracerebroventricular injection, resulting in a modest, but significant, extension of survival (Figure 2A) (26). Combinatorial treatment with AAV9-PLS3 did not significantly increase survival with the 1-nmol dose of MOE1v11 (Figure 2A). However, the presence of one longer-lived animal in the combined treat-
ment group highlights that 1 nmol MOE1v11 might be approaching a threshold at which the beneficial effects of PLS3 could be observed. AAV9-PLS3 administration was repeated; however, in this cohort the concentration of MOE1v11 ASO was increased 2-fold by injecting 2 nmol MOE1v11 (Figure 2B). As anticipated, the cohort of SMA mice receiving only MOE1v11 ASO lived longer than the untreated SMA mice, averaging 30.6 ± 3.8 days, consistent with an increase in total SMN levels (Figure 2, B and C). In contrast to the lower dose of ASO, the 2-nmol treatment group cotreated with AAV9-PLS3 achieved significantly improved life spans, averaging 43.8 ± 4.3 days with MOE1v11 plus AAV9-PLS3 (Figure 2B). Similar to what was observed in the Smn2B/– mice treated with ASO and AAV9-PLS3, combination treatment did not significantly improve weight gain compared with treatment with ASO (Figure 2D). Collectively, this is the first demonstration to our knowledge of two potential therapeutic modalities that improve survival in an SMA model, demonstrating the potential utility of SMN-independent and SMN-dependent combinatorial approaches. Subsequent combinatorial experiments will focus exclusively on AAV9-PLS3 in combination with the coadministration of 2 nmol MOE1v11.

To determine whether an improvement in life span correlated with functional improvements, time-to-right assays were performed on the various cohorts of SMA mice. In severe SMA mouse models, the ability of SMA mice to turn over when placed on their backs and the time at which they turn is a sensitive measurement of the phenotype and an assessment of gross motor ability (27). Time-to-right assays were performed on mice in the following groups: SMA untreated; SMA treated with MOE1v11 alone; or SMA treated with MOE1v11 plus AAV9-PLS3. We focused our analysis between P14 and P23, a 10-day window in which phenotypic differences become evident between treatment conditions. Times to right of mice in the dual-treatment group (MOE1v11 plus AAV9-PLS3) were consistently faster than those treated with MOE1v11 alone during this window and reached statistical significance (P < 0.05 for P14 and P20) at P20 (Figure 3A). Because the time-to-right assay was terminated at 30 seconds, this test fails to account for differences in motor function if a significant number of mice fail to right. Thus, we analyzed the number of times each mouse fails to right between P14 and P23 when tested once daily (Figure 3B). Mice treated with MOE1v11 plus AAV9-PLS3 exhibited significantly fewer failed righting attempts compared with untreated SMA mice or SMA mice treated with MOE1v11 alone.

The improved motor function exhibited by the dual-treatment group suggests that vector-mediated delivery of PLS3 improved muscle function potentially by reducing the severity of skeletal muscle atrophy. To determine if the MOE1v11 plus AAV9-PLS3 treatment significantly reduced skeletal muscle atrophy compared with MOE1v11 treatment alone, the gastrocnemius muscle was analyzed. The gastroc-
nemius muscle was removed from P12 mice, cryosectioned, and stained with laminin to label the cell membranes (Figure 4A). Mean muscle fiber area from cross sections was quantitated, revealing that MOE1v11 plus AAV9-PLS3 treatment resulted in a modest but significant improvement in mean muscle fiber area (Figure 4B) and shifted the distribution frequency of muscle fiber area closer to unaffected muscle distribution patterns (Figure 4C).

Transgenic overexpression of PLS3 in severe SMA mice has recently been shown to improve the integrity of affected NMJs (9, 28). PLS3 acts by increasing actin bundling, which is thought to correct cytoskeletal defects that have been implicated in NMJ pathology in SMA. Additionally, in vitro experiments have identified a role of PLS3 in generating actin-based force generation, indicating a potential role in neuritogenesis (29). Increasing SMN levels with MOE1v11 treatment alone has also been shown to improve NMJ pathology (26). To determine whether dual treatment ameliorates the NMJ defects compared with MOE1v11 monotherapy, treatment groups were analyzed by immunofluorescent staining of the longissimus capitis muscle. This muscle was selected because prior studies have demonstrated that the longissimus capitis muscle is particularly sensitive and exhibits significant pathology with respect to NMJ maturation, denervation, and morphology in the SMNΔ7 mouse model (30). Both treatment groups resulted in visibly improved NMJ size and innervation compared with the untreated SMNΔ7 mouse (Figure 5, A and B), as evidenced by the similar levels of fully innervated NMJs as well as the degree of partial and fully denervated end plates (Figure 5C). In contrast, the dual-treatment group exhibited significantly larger motor end plates compared with SMNΔ7 mice treated exclusively with the MOE1v11 ASO (Figure 5D). The dual treatment also improved NMJ maturation, as evidenced by a significant improvement in the number of end plate perforations compared with mice treated with ASO alone (Figure 5E).

Despite improvements in NMJ size and maturation, codelivery of MOE1v11 and PLS3 did not significantly increase the motor neuron count or size of motor neuron cell bodies in the L4–L6 ventral root (Supplemental Figure 1, A–C; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.89970). Consistent with the observation that codelivery of MOE1v11 and PLS3 did not increase fully innervated NMJs, these data suggest that the observed improvements in motor function and muscle atrophy are likely due in part to modifications of the synaptic architecture as opposed to an improvement in motor neuron survival and innervation.
Discussion

The search for disease modifiers beyond SMN2 has been ongoing; however, the potential role of putative protective genetic modifiers of SMA, including PL3S, has been controversial. The controversy stems from results generated in a variety of important models of SMA that have resulted in conflicting conclusions. In support of PL3S as a protective modifier is evidence that indicates that (a) clinically and genetically, PL3S correlated with decreased disease severity in discordant SMA siblings (15); (b) PL3S transient expression in Smn-deficient zebrafish rescued motor axon defects (15); (c) transgenic expression of PL3S ameliorates the NMJ pathology in severe SMA mice (12); and (d) homozygous PL3S transgenic SMA mice respond dramatically to low doses of ASO, resulting in a significant extension of survival and improved NMJ pathology (31). In contrast to the beneficial effects of PL3S on SMA-associated pathology in the models above, transgenic expression in the SMNΔ7 background failed to extend survival and failed to decrease disease severity in the SMNΔ7 background (32). One interpretation of these conflicting results is that SMN-independent pathways, such as PL3S, are not able to compensate for SMN deficiencies that lead to severe SMA; however, when SMN levels are less depleted, PL3S is able to decrease disease severity. Essentially, PL3S is capable of modifying disease, provided the disease context is relatively mild.

The notion that PL3S can modify less severe models of SMA is consistent with the current report:

Figure 3. AAV9-PL3S improves motor function in SMNΔ7 mice treated with 2 nmol MOE1v11. (A) Mean time to right ± SEM between P14 and P23. Student’s 1-tailed t test was applied between mice treated with MOE1v11 and AAV9-PL3S and MOE1v11-treated mice on each day. (B) Scatter plot of failure events per mouse when assayed once daily between P14 and P23 for the ability to right. Line represents mean ± SEM. One-way ANOVA with Newman-Keuls post-hoc test was applied. (*P < 0.05; ***P < 0.001; NS, P ≥ 0.05).
vector-mediated delivery of \textit{PLS3} failed to modify the severe SMNΔ7 phenotype, yet the same dose of AAV9-\textit{PLS3} significantly extended survival in an intermediate model of SMA, \textit{Smn}2B/– mice. Additionally, by coadministering a low dose of the MO E1v11 ASO with AAV9-\textit{PLS3}, this effectively created an intermediate phenotype. In this ASO-mediated intermediate model, \textit{PLS3} was able to significantly reduce disease severity, as evidenced by the increased life span, improved mobility, and NMJ pathology. As we have conducted this analysis using AAV9-mediated transduction, these results give additional support to the therapeutic potential of \textit{PLS3} as a target for combinatorial therapy in human patients with SMA. As \textit{PLS3} is X-linked and has only been described as a modifier in type III and IV female patients (15), the possibility exists that a compound that increases \textit{PLS3} transcription, stability, or activity might be efficacious in some types of SMA. It is not clear whether responses to \textit{PLS3} induction would be restricted by gender. Currently, the human examples have demonstrated that the protective activity observed with increased \textit{PLS3} is restricted to females; however, this was not the case in zebrafish and mice (12, 15, 31). In our results using a vector to deliver \textit{PLS3}, we did not detect a difference regarding gender-based responses to \textit{PLS3} treatment, consistent with the transgenic models.

Beyond the therapeutic potential of \textit{PLS3}, the ability of \textit{PLS3} to partially rescue the SMA phenotype suggests that one of the important functions related to SMA development centers around actin dynamics. While it is clear that SMN performs critical roles in a variety of RNA-processing pathways, delineating a
single gene or a single pathway that was responsible for the SMN-associated development of disease has been challenging. Clearly, PLS3 does not completely reverse the devastating disease phenotype observed in severe SMA mice, nor does it fully prevent disease development in milder mouse models of SMA. Therefore, the possibility exists that other SMN functions are responsible, either individually or through interaction with other targets in yet unknown ways, and give rise to the complete SMA pathology. It is likely that additional SMN-independent modifiers exist, and, like with PLS3, these genes may shed light upon important functional pathways related to SMA development and may provide new targets for therapeutic development that can be used as stand-alone therapies or in combination with SMN-dependent therapeutics. These results, as well as those in several other studies, highlight that proteins involved in actin dynamics are also potential targets for combinatorial therapeutics.

Figure 5. AAV9-PLS3 improves neuromuscular junction size and maturation in SMNΔ7 mice treated with 2 nmol MOE1v11. Longissimus capitis muscles were harvested from mice receiving various treatments on P12 and were labeled with α-bungarotoxin (red), synaptophysin (green), and neurofilament (green). (A) Immunofluorescence labeling demonstrating neuromuscular junction (NMJ) architecture and innervation (original magnification, ×20). (B) Immunofluorescence labeling revealing degree of end plate maturation (original magnification, ×20). (C) Mean number of innervating axons ± SEM. (D) Scatter plot representing mean end plate area ± SEM. (E) Scatter plot representing the number of NMJ perforations ± SEM. One-way ANOVA with Newman-Keuls post-hoc test was applied (*P < 0.05, **P < 0.01, ***P < 0.001, NS, P ≥ 0.05). NMJs were taken from 3 SMNΔ7 mice and 4 mice from all other groups and pooled as follows: untreated SMNΔ7, n = 89; 2 nmol MOE1v11, n = 118; 2 nmol MOE1v11 AAV9-PLS3, n = 128; unaffected, n = 155).
Methods

Animal procedures and delivery of therapeutics. All mice were stored in pathogen-free conditions. Genotype was determined on P1 by tail clip, tissue lysis, and PCR. The SMN7 colony (stock no. 005025; Jackson Laboratory) was maintained by heterozygous breeder pairs (mSmm7−/−,SMN2+/−,SMN7+/+). The same pairs were used to breed SMA mice. The Smm7−/− mice were bred from two colonies: mSmm7−/− heterozygotes (stock no. 006214; Jackson Laboratory) and Smm28/28 homozygotes (C57BL/6 background; gift from Rashmi Kothary, Ottawa, Ontario, Canada). All injections were performed at the end of P1. Viral delivery was performed by intraventricular injection into the superficial facial vein. MOE1v11 delivery was performed by intracerebroventricular injection using methods described previously (33, 34). Time-to-right assays were performed between P14 and P23 by placing the animal on its back and timing its ability to turn over and stand on all 4 legs, with 30 or more seconds to right qualifying as a failure. All mice were fed low-fat stock diets (Harlan Teklad 8640) except for mice in the 2 nmol MOE1v11 plus AAV9-PLS3 survival study, which were fed high-fat breeder diets (PicoLab Mouse Diet 20 — 5058, LabDiet).

Production and purification of AAV9-PLS3 vector. 293T HEK cells (ATCC CRL 3216) were cultured in four 10-floor cell factories until approximately 85% confluent. Cells were triple transfected with Rep2Cap9 (a gift from James Wilson, University of Pennsylvania, Philadelphia, PA) (serotype 2 Rep proteins), pHelper (adenovirus helper constructs; Stratagene), and scAAV-CBA-PLS3 using 25-kDa polyethyleneimine at a molar ratio of 1:1:1. Media were changed 24 hours after transfection, and cells were harvested at 48 hours after transfection. Cells were suspended in 10 mmol Tris, pH = 8.0, lysed by 5 freeze-thaw cycles in liquid nitrogen, DNAse treated, and protease treated. CsCl crystals were added to the lysate (0.631 g of CsCl per ml of the lysate) to generate a solution with a density of approximately 1.4 mg/ml. After incubation at 37°C for 45 minutes, the solution was centrifuged at 3,184 g and 3,184 μl of liquid nitrogen. Virus was purified from lysate by 3 rounds of density gradient centrifugation at an average RCF of 158,000. High-titer fractions were detected after each round of centrifugation using quantitative real-time PCR modified from a previously described protocol (35). The final fractions were dialyzed exhaustively against PBS and stored at 4°C until use.

MOE1v11 ASO therapeutic. MOE1v11 was synthesized with morpholinom modifications at every nucleotide position (GeneTools L.L.C.). MOE1v11 is a 20-nucleotide oligomer (5′-CUAUAAUGUAAUUCAGA-3′).

Histological analysis and quantification of gastrocnemius muscle fiber area. Three animals from each treatment and control groups at age P12 were randomly selected and anesthetized by anesthetic inhalant Isoflurane USP, VetOne (1-chloro-2, 2,2-trifluoroethyl difluoromethyl ether; 50 mg/kg), followed by transcardiac perfusion with PBS solution, and then were fixed with 4% paraformaldehyde (Sigma-Aldrich). The gastrocnemius was dissected and subsequently flash-frozen in liquid nitrogen–cooled isopentane. Frozen muscle tissue was then cryosectioned at 20 μm per section. Immunohistochemistry was modified from a previously described protocol (36). Tissues were labeled using anti-laminin (1:250; catalog L9393, Sigma-Aldrich). Samples were mounted using Vectashield (Vector Laboratories Inc.) containing DAPI to visualize nuclei. Fiber area quantification was performed using ImageJ software (NIH). 240 muscle fibers were analyzed per mouse and grouped by condition. Statistical analysis was performed using a 1-tailed Student’s unpaired t test.

Immunohistochemistry of NMJs. Three to four animals from each treatment and control group at age P12 were randomly selected and anesthetized by anesthetic inhalant Isoflurane USP, VetOne (1-chloro-2, 2,2-trifluoroethyl difluoromethyl ether; 50 mg/kg) followed by transcardiac perfusion with PBS solution, and then were fixed with 4% paraformaldehyde (Sigma-Aldrich). Whole-mount preparations were performed by dissecting and examining the longissimus capitis muscle. Tissues were stained using specific antibodies, including anti-neurofilament (1:2,000; catalog AB5539, Chemicon, EMD Millipore) and anti-synaptophysin (1:200, catalog YE269, Life Technologies). Acetylcholine receptors were labeled with Alexa Fluor 594–conjugated α-bungarotoxin (Life Technologies). NMJ analysis was performed on at least 3 randomly selected fields of view per muscle per mouse (<40 objective; Leica DM5500 B, Leica Microsystems Inc.). Muscle preparations were imaged using a laser scanning confocal microscope (<40 objective; Leica TCS SP8, Leica Microsystems Inc.).

Statistics. Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software Inc.) and Microsoft Excel version 15.19.1 (Microsoft Corporation). Data were analyzed using the log-rank Mantel-Cox test for survival comparisons, Student’s 1-tailed t test for time-to-right assay, and 1-way ANOVA with Newman-Keuls post-hoc test for all other analyses. A P value of less than 0.05 was considered significantly significant.
Study approval. Animals were housed and treated in accordance with the Animal Care and Use Committee guidelines of the University of Missouri. The Animal Care and Use Committee of the University of Missouri approved these studies.

Author contributions
KAK, EV, EYO, IJG, and CLL designed and performed experiments, analyzed the data, and contributed to the writing of the manuscript. LLA and DDWC provided reagents/materials and performed experiments.

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Address correspondence to: Christian L. Lorson, Department of Veterinary Pathobiology, Christopher S. Bond Life Sciences Center, 1201 Rollins, Room 471G, University of Missouri, Columbia, Missouri 65211-7310, USA. Phone: 573.884.2219; E-mail: lorsonc@missouri.edu.


