



Review

Gene Therapy for Metachromatic Leukodystrophy

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Leukodystrophies (LDs) are rare, often devastating genetic disorders with neurologic symptoms. There are currently no disease-specific therapeutic approaches for these diseases. In this review we use metachromatic leukodystrophy as an example to outline in the brief the therapeutic approaches to MLD that have been tested in animal models and in clinical trials, such as enzyme-replacement therapy, bone marrow/umbilical cord blood transplants, ex vivo transplantation of genetically modified hematopoietic stem cells, and gene therapy. These studies suggest that to be successful the ideal therapy for MLD must provide persistent and high level expression of the deficient gene, arylsulfatase A in the CNS. Gene therapy using adeno-associated viruses is therefore the ideal choice for clinical development as it provides the best balance of potential for efficacy with reduced safety risk. Here we have summarized the published pre-clinical data from our group and from others that support the use of a gene therapy with AAVrh.10 serotype for clinical development as a treatment for MLD, and as an example of the potential of gene therapy for LDs especially for Krabbe disease, which is the focus of this special issue. © 2016 Wiley Periodicals, Inc.

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Leukodystrophies (LDs) are a group of >30 heterogeneous genetic disorders affecting the white matter of the central (CNS) and/or the peripheral (PNS) nervous system and include, among many others, the more common metachromatic leukodystrophy (MLD) and globoid cell leukodystrophy (Krabbe's disease; Parikh et al., 2015; Vanderver et al., 2015). Most LDs, especially in the pediatric population, lead to motor deficits that often dominate the clinical presentation (Parikh et al., 2015). The LDs have variable clinical manifestations that include walking difficulties, spasticity and/or ataxia, and behavioral and cognitive decline with different degrees of penetration even for the same genetic defect (Gordon et al., 2014; Parikh et al., 2015). Based on an understanding of

the pathogenesis of the LDs, a variety of approaches toward therapy has been tried, including enzyme replacement therapy, bone marrow and umbilical cord stem cell transplantation, and gene therapy (Helman et al., 2015; Aubourg, 2016; Choudhury et al., 2016). As an example of the strategy for development of therapy for the LDs, we review the use of in vivo adeno-associated virus (AAV)-mediated CNS gene therapy for MLD, one of the most common LDs. We conclude this Review by summarizing how these findings relate to the work being done in developing treatments for Krabbe's disease.

MLD

MLD is an autosomal recessive lysosomal lipid storage disorder caused by deficiency of the lysosomal arylsulfatase A (ARSA) enzyme (von Figura et al., 2001; Aubourg et al., 2011; Batzios and Zafeiriou, 2012) or, more rarely, a deficiency of its activator protein saposin B (SAP-B; Holtschmidt et al., 1991). The estimated incidence of MLD ranges from 1.4 to 1.8/100,000 live births (Gieselmann and Krageloh-Mann, 2010; Aubourg et al., 2011). Most subjects with MLD are of caucasian origin (Cesani et al., 2016). The arylsulfatase A gene is located on chromosome 22q13.33 (Narahara et al., 1992) and is alternatively spliced with eight or nine exons combining to produce three different mRNA species (Kreysing et al., 1990). These encode two isoforms of the same protein, an aryl sulfatase involved in the lysosomal degradation of sphingolipid cerebroside 3-sulfate ("sulfatide"; Kreysing et al., 1990; von Figura et al., 2001). In subjects inheriting two mutant *ARSA* genes, sulfatides

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accumulate in microglia, oligodendrocytes, and Schwann cells, resulting in widespread demyelination in the CNS and PNS (Gieselmann, 2003; Molander-Melin et al., 2004; Takahashi and Suzuki, 2012; Patil and Maegawa, 2013). Sulfatides also accumulate in CNS neurons, contributing to additional neuronal dysfunction and degeneration (Gieselmann et al., 1998; Gieselmann, 2003; Molander-Melin et al., 2004; Wittke et al., 2004; Sevin et al., 2007a). At the cellular level, MLD is characterized by progressive loss of glia and neurons (von Figura et al., 2001; Molander-Melin et al., 2004; Wittke et al., 2004). Undigested sulfatides accumulate in other tissues, but this does not contribute significantly to the pathogenesis of MLD (Gieselmann, 2003). As a major component of the myelin membrane, sulfatides are essential to its structure and function. Excess sulfatide triggers an inflammatory response, including microglial activation, astrogliosis, recruitment of peripheral macrophages, and secretion of proinflammatory cytokines, leading to apoptosis of glia and neurons (Sevin et al., 2007a; Patil and Maegawa, 2013).

The definitive diagnosis of MLD requires a comprehensive evaluation based on a wide set of diagnostic procedures, including biochemical and molecular tests and neuroradiological (gray matter volume loss, abnormalities in white matter) and neurophysiological evaluations. The disease is typically classified according to the age of onset (von Figura et al., 2001; Sevin et al., 2007a). The late-infantile form, which is the most frequent (approximately 50%; Cesani et al., 2016), usually manifests in the second year of life. The juvenile variant, with an onset between age 4 and 12 years, is further subdivided into early-juvenile and late-juvenile forms depending on whether the onset is before or after 6 years of age. The term adult MLD refers to patients with onset of neurological symptoms after the age of 12 years (von Figura et al., 2001). These different forms of MLD are dependent on the inherited mutation and the resultant levels of ARSA activity.

CLINICAL MANIFESTATIONS

Clinically, signs and symptoms caused by the involvement of CNS and PNS characterize the various forms of MLD. Age of MLD onset varies from 18 months to adulthood, and in the majority of cases the prognosis is severe, leading to a vegetative stage or death within a few years of diagnosis (von Figura et al., 2001; Aubourg et al., 2011). The natural history of clinical disease progression for all forms of MLD has not yet been defined. However, the neurologic symptoms of patients with classical late-infantile or early-juvenile forms of MLD are well known, with little variation in the age of onset of symptoms (Sevin et al., 2007a; Groeschel et al., 2011). Nearly all patients with the most severe form of late-infantile MLD are tetraplegic and in a vegetative stage before the end of the second year (Gieselmann and Krageloh-Mann, 2010). Similarly, most patients with early-juvenile form of MLD are tetraplegic and in a vegetative stage 12–18 months after the onset of the first symptoms (Gieselmann and

Krageloh-Mann, 2010). Among these symptoms, the most common form of MLD involves late-infantile presentation, with muscle wasting, weakness, muscle rigidity, developmental delays, and progressive loss of vision, seizures, impaired swallowing, paralysis, and dementia (Biffi et al., 2008; Gieselmann and Krageloh-Mann, 2010). Motor deterioration (loss of acquired motor skills) may present itself as early as 12 months in late-infantile MLD cases, beginning with a slowing in the development of motor skills in infants. Death occurs in early childhood.

GENETICS

In addition to the age of onset and symptoms, genotype is also a predictor of the phenotype and the severity of disease, particularly in the late-infantile forms (Cesani et al., 2016). More than 150 *ARSA* mutations have been described to date according to the Human Gene Mutation Database (HGMD; <http://www.hgmd.cf.ac.uk/ac/index.php>). In addition, rare mutations in the activator protein saposin B have also been described, which also cause MLD (Rafi et al., 1990; Zhang et al., 1990; Holtschmidt et al., 1991; Cesani et al., 2016). For late-infantile MLD, the most common mutation is an *ARSA* splicing defect, c.465 + 1G > A, and for adult-onset MLD the most common is c.1283C > T; together these mutations account for almost 33% of MLD alleles (Cesani et al., 2016). Although data on genotype/phenotype correlations are limited, it is known that, if a subject is homozygous for mutations that do not make any functional *ARSA*, then they will have the severe late-infantile form of the disease. Alternatively, if one of the alleles is associated with residual enzyme activity, it partially mitigates the disease and leads to the juvenile form of the disease. Finally, if the subject is homozygous for two alleles, both of which lead to some *ARSA* activity, then it is likely that this will lead to a late-juvenile or even adult form of MLD (Gieselmann and Krageloh-Mann, 2010). In general, as might be expected, the greater the *ARSA* activity, the later the onset of disease. However, there is significant clinical variability in the late-onset patients even if they are siblings harboring the same *ARSA* genotype, suggesting that other genetic or epigenetic factors might influence the course of MLD progression (Berger et al., 1997; Biffi et al., 2008).

PATHOPHYSIOLOGY

The severe atrophy in the CNS and PNS in MLD correlates with sulfatide accumulation, which appears as dense granules of accumulated lysosomal storage material (Gieselmann, 2003; Molander-Melin et al., 2004; Takahashi and Suzuki, 2012; Patil and Maegawa, 2013). Sulfatide accumulation in vitro can trigger inflammatory cytokines, which in turn are thought to be involved in apoptosis (Constantin et al., 1994; Laudanna et al., 1994; Sevin et al., 2007a). In the CNS, there is also a decrease in galactosylceramide in MLD-affected patients (von Figura et al., 2001; Sevin et al., 2007a). Galactosylceramide and sulfatide are the two major glycosphingolipids of the

myelin sheath, accounting for 23% and 6% of total lipids, respectively (Taylor et al., 2004). Both essential lipids are associated with the extracellular surface of the myelin sheath via Ca^{2+} -mediated interactions and contribute to the stability, flexibility, and compaction of myelin (Ohmi et al., 2003). Aberrant storage granules have also been observed in the kidney, gallbladder, liver, pancreas, pituitary gland, adrenal cortex, retina, testes, sweat glands, and rectal tissues (Gieselmann et al., 1998; Biffi et al., 2008; Patil and Maegawa, 2013). Unlike the CNS, the functions of most of these tissues (except for the gall bladder, which also accumulates high levels of sulfatides and whose dysfunction may be an indicator of MLD) are not affected by sulfatide accumulation, at least during the life of those with MLD (Gieselmann et al., 1998; von Figura et al., 2001; Agarwal and Shipman, 2013). A mouse model of MLD was generated by Hess et al. (1996), in which they knocked out *ARSA* gene expression by insertion of a neomycin cassette in exon 4. Although the mice show all the hallmarks of the human disease (sulfatides accumulation in neuronal and nonneuronal tissues, axonal size reduction, astrogliosis, acoustic ganglion neuron loss, and altered Purkinje cell morphology), they do not have a shortened life span, nor do they display white matter defects (Hess et al., 1996). These mice do not express functional *ARSA* enzymes, so the model is equivalent to the late-infantile form of MLD and has been used over the past 2 decades to search for a cure for MLD.

THERAPEUTIC APPROACHES TO MLD

Various therapeutic approaches to MLD have been tested in experimental animal models. Among these are several promising approaches with clinical translation potential including 1) enzyme replacement therapy (ERT), 2) bone marrow transplants (BMT), 3) gene therapy by ex vivo transplantation of genetically modified hematopoietic stem cells (HSC), and 4) AAV-mediated gene therapy directly to the CNS. The following sections briefly describe the first three strategies, followed by a detailed description of AAV-mediated gene therapy directly to the CNS.

ERT

ERT has been successful in preventing or reversing the systemic manifestations of several lysosomal disorders by taking advantage of the ability of the cell to take up infused enzyme via the cation-independent mannose-6-phosphate (M6P) receptors present on the surface of virtually all cells (Funk et al., 1992; Ghosh et al., 2003; Willingham et al., 1981). Intravenous administration of recombinant *ARSA* (40 mg/kg) in a mouse model of MLD reduced the sulfatide storage in peripheral tissues, including the sciatic nerves (Matzner et al., 2005). This therapeutic approach might be used to prevent or reverse demyelination in the peripheral nerves of MLD patients. ERT also reduced the sulfatide storage in the brain of MLD mice, an unexpected finding insofar as the blood-brain barrier prevents direct transfer of *ARSA* enzyme to

the CNS from the circulation (Matzner et al., 2005). In an effort to explore the “window of opportunity” for ERT in a more severe MLD mouse model, Matthes et al. (2012) developed a double-transgenic *ARSA*^{−/−} mouse, with immunotolerance to human *ARSA* and supranormal rates of sulfatide synthesis, and treated the mice at various ages with weekly injections of recombinant *ARSA* (20 mg/kg i.v. for 16 weeks). They found improvements in MLD symptoms (e.g., reduction in sulfatide storage) in only the mice treated at early time points (4 months) and not with treatment at later time points (8–12 months), suggesting that i.v. ERT will likely only be efficacious in patients treated early. Additional studies by this group using these double-transgenic MLD mice and ERT explored continuous intrathecal infusion of recombinant *ARSA* (average of 4.3 $\mu\text{g/day}$ for 4 weeks) using minipumps to evaluate bypassing the blood-brain barrier for ERT (Stroobants et al., 2011). This study showed complete reversal of sulfatide storage in the infused hemisphere and correction of various measures of CNS dysfunction, suggesting that continuous infusion of a low dose of *ARSA* may be efficacious for the treatment of MLD. However, the extent of metabolic correction with ERT unlikely will be sufficient and in time to arrest the rapid cerebral demyelinating process that occurs in aggressive and devastating forms of infantile and early-juvenile MLD. Furthermore, because the blood-brain barrier restricts access to the CNS of most large proteins in the blood compartment, it is unlikely that ERT will work unless delivered directly to the CNS (Abbott, 2013; Muldoon et al., 2013). Given that the half-life of *ARSA* is approximately 4 days (Matzner et al., 2005), frequent, repeated delivery of the therapeutic protein to the CNS is required. Current ERT clinical trials are aimed at increasing the dosage and frequency to overcome the short-half life of the *ARSA* enzyme (Matzner et al., 2005). One such ongoing trial with ERT for treatment of MLD is the IDEAMLD phase I/II clinical trial (NCT01510028), which uses multiple intrathecal infusions with recombinant *ARSA* (HGT-1110; Shire Human Genetic Therapies) to translate the mouse studies of Stroobants (2011) into clinical use. No preliminary results are currently available.

Bone Marrow and Umbilical Cord Blood Transplantation

One strategy to treat MLD is to transplant cells secreting *ARSA*. Studies in bone marrow transplantation models in GFP⁺ transgenic mice showed that male donor transplanted cells (6×10^6 cells/mouse) could be detected in female recipient brains (Brazelton et al., 2000; Mezey et al., 2000). Bone marrow transplantation of HSCs ($2\text{--}3 \times 10^6$ cells i.v.) increased the number of donor cells in the wild-type C57Bl/6J recipient CNS, with evidence that macrophages could differentiate into microglia (Eglitis and Mezey, 1997). Furthermore, when GFP-tagged bone marrow cells were transplanted into wild-type C57Bl/6 mice, HSC-derived microglia cell engraftment was enhanced by the local

neuropathology, allowing healthy microglia to replace damaged microglia in up to 25% of the regional population (Priller et al., 2001). With regard to clinical trials, there have been multiple reports of MLD patients gradually improving their motor and behavioral function scores following umbilical cord blood transplants (UCBT) but mixed results for allogeneic HSCT (Krivit et al., 1999a; Pierson et al., 2008; Smith et al., 2010; Cable et al., 2011; de Hosson et al., 2011; Martin et al., 2013; van Egmond et al., 2013; Chen et al., 2016; Helman et al., 2015). The early-onset forms of MLD (LI) are problematic for successful HSCT or UCBT even when the patients are still presymptomatic because of the rapidity of disease progression (Bredius, 2007; Martin, 2013). In one long-term study, 60% of the LI-MLD children treated by UCBT survived to the 5-year followup; however, only asymptomatic children benefitted, whereas the children symptomatic at the time of transplant all died (Martin, 2013). The results with UCBT in later/less-severe forms of MLD are more promising. One recent report of a patient with juvenile MLD, who underwent an allogeneic cord blood transplant with 100% donor cell engraftment, had subsequent MRI scans showing clear improvement of white matter abnormalities 18 months posttransplant (van Egmond et al., 2013). In one UCBT study, the long-term progress of three asymptomatic children (aged 2–5 years) was compared with that of untreated siblings with symptomatic early-juvenile MLD. In followup examinations after transplantation (at 6 and 14 years), the transplant procedure appeared to slow the progression of the disease significantly (Chen et al., 2016).

BMT of Genetically Modified Autologous HSC

A therapeutic strategy that could make stem cell therapy a more viable option for MLD combines HSC transplants with gene therapy (Biffi et al., 2013). Specifically, this strategy involves transplantation of autologous HSC genetically corrected by retrovirus or lentivirus to express ARSA. Studies with HSC modified with a murine retrovirus vector (1.5×10^6 cells i.v. in 5–6-week-old mice) showed increased sulfatide reduction but no effects on the disease progression in MLD mice (Matzner et al., 2000, 2002). Matzner et al. hypothesized that very high levels of ARSA enzyme might be required for correction of the lysosomal defect in the CNS of MLD mice, and, because γ -retroviruses do not transduce nondividing cells, larger number of cells would have to be transduced to obtain these high levels of ARSA expression. In contrast, studies with a lentiviral vector to modify the HSC had significant impact on the MLD mice (1×10^6 cells i.v. in 6-week mice; Biffi et al., 2004, 2006). There was extensive repopulation of CNS microglia and PNS macrophages by the transgene-expressing cells, and the recruitment of these HSC-derived cells was rapid and robust (Biffi et al., 2004). Importantly, there was overexpression of ARSA enzyme in bone marrow-derived brain microglia (Biffi et al., 2006). In clinical studies, this ex vivo gene therapy approach uses the readministration of a patient's genetically corrected bone marrow stem cells. In recent clinical trials, autologous

CD34⁺ HSCs transduced with a human-ARSA encoding lentiviral vector, underwent rounds of selection to isolate and expand the most viable clones, which were readministered (2×10^6 cells/kg i.v.) in presymptomatic late-infantile MLD and pre- or early symptomatic early-juvenile MLD patients (NCT01560182; ClinicalTrials.gov). This “TIGET-MLD” trial in Italy (phase I/II) was started several years ago and is in the posttherapy monitoring phase, with the primary completion date in April, 2018. In a recent published report, this lentiviral-based approach for MLD gene therapy has demonstrated efficacy in the first three patients who were treated in the presymptomatic phase (Biffi et al., 2013; Aubourg, 2016). These patients have sustained, above-normal ARSA activity in cerebrospinal fluid samples, and the natural history of MLD disease progression appears to be halted (Biffi et al., 2013). This is a promising therapy for subjects who were identified at a presymptomatic stage from families with a prior family history of MLD (such as an affected sibling), but it has not been shown to be efficacious when the disease has already clinically manifested. Unfortunately, a large majority of the new MLD cases are from families with no prior family history of MLD, and genetic screening for MLD is not included in the newborn genetic screening tests (Ombrone et al., 2016). Hence, most children with severe forms of MLD would not be diagnosed at the presymptomatic phase of the disease, making it unlikely for this therapeutic option to be effective for many MLD patients.

Overview of AAV-Mediated CNS Gene Therapy

A method by which the genetic message for ARSA gene could be delivered directly to the CNS to provide long-term, sustained, and persistent correction without disruption of endogenous genes would be an ideal alternative to treat MLD. AAV-based gene therapy uniquely meets these requirements. First, the therapeutic agent can be delivered directly into the CNS via intraparenchymal, intracerebroventricular, and intracisternal routes and thereby express the ARSA protein locally on a persistent, permanent basis, following a one-time administration. Second, comparisons of genotype and phenotype suggests that only 5–10% of normal enzyme activity is sufficient to prevent disease symptoms (von Figura et al., 2001; Rauschka et al., 2006), levels that are likely achievable by AAV-mediated gene expression of ARSA (Rosenberg et al., 2014; Golebiowski et al., 2015). Third, the ARSA protein is secreted and is capable of cross-correcting neighboring cells. Thus, it is not necessary to transfer the normal ARSA cDNA to all of the cells in the CNS; corrected cells will secrete ARSA protein, which is then endocytosed via the M6P receptor pathway (Matzner and Gieselmann, 2005) by neighboring cells for therapeutic correction. Therefore AAV-based gene therapy, which would unlikely transduce every cell with the normal therapeutic gene, could be the source of corrective enzymes for even the noninfected neighboring cells and thus provide a greater potential for success of the therapy. This is of particular importance for oligodendrocytes, which cannot be transduced in vivo by

most gene therapy vectors, including most serotypes of AAV (Cearley et al., 2008; Lawlor et al., 2009). In addition, axonal connections provide extension to the range of cells receiving the ARSA protein, well beyond the site of vector administration (Luca et al., 2005; Sevin et al., 2006). These concepts, together with the knowledge that AAV vectors have low immunogenicity and low oncogenic potential via insertional mutagenesis and the availability of methods to manufacture the amounts of vector needed for dosing needs for large clinical studies make them ideal for this application.

The early studies with AAV vectors utilized AAV2, a human-derived AAV serotype that mediates persistent but low-level expression with relatively slow onset (Xiao et al., 1997; Lo et al., 1999; Tenenbaum et al., 2004). As additional human-derived AAV vectors were developed, including serotypes 1, 5, and 6, it became apparent that different AAV capsid serotypes differed with respect to target specificity and cell tropism, rate of onset of expression, extent of expression, and ability to circumvent pre-existing anti-AAV immunity (Vite et al., 2003; Burger et al., 2004; Taymans et al., 2007; Calcedo et al., 2009; Ciron et al., 2009; Boutin et al., 2010; Asokan et al., 2012; Calcedo and Wilson, 2013). The spectrum of AAV serotypes was further expanded with the discovery of nonhuman primate AAV serotypes, including AAVs 8, 9, and rh.10 (Gao et al., 2005). Our initial studies with AAV-mediated gene therapy for MLD were conducted with AAV5, and then, with the discovery of the nonhuman primate derived serotypes, our second-generation studies were carried out with AAVrh.10 (Sondhi et al., 2007, 2008; Piguet et al., 2012; Sondhi et al., 2012; Rosenberg et al., 2014). Our choice of AAVrh.10 was based on the successful use of this vector for treatment of the mouse model of late-infantile neuronal ceroid lipofuscinosis (LINCL), another lysosomal storage disease, and the translation of these studies to a clinical study (Sondhi et al., 2007, 2008, 2012). Below we describe the initial studies with AAV5 and the rationale for and the followup studies with AAVrh.10.

AAV5-Mediated Gene Therapy for MLD

In 2006, Sevin et al. demonstrated that intraparenchymal administration of an AAV5 vector expressing the human *ARSA* gene (3×10^9 particles/mouse) to the CNS (cerebellar vermis and internal capsule) of the MLD mouse model at 3 months of age (presymptomatic) led to rapid, widely distributed, and persistent expression of the functional ARSA protein throughout the brain to at least 15 months postinjection (Sevin et al., 2006). Analysis of the vector genome and ARSA distribution gave evidence for in vivo cross-correction of many neurons and astrocytes not directly transduced by the vector, demonstrating that the widespread ARSA expression was mostly a result of the dispersal of the enzyme. Importantly, ARSA delivery reversed sulfatide storage, a hallmark of the disease, and prevented neuropathological abnormalities (including glycolipid storage, microglial activation and gliosis, neuro-

nal degeneration) and neuromotor impairment associated with the disease. The mouse model of MLD does not recapitulate the severity of human symptoms, so there are no early deaths from the ARSA deficiency in MLD mice; therefore, no survival studies were performed to assess the impact of AAV5-ARSA administration on this. Subsequent studies, utilizing the same vector, dosage (3×10^9 particles), and intraparenchymal sites, demonstrated that direct administration of the AAV5 vector into the brain of the MLD mouse model at a symptomatic stage (6 months) results in sustained expression of ARSA and prevention of sulfatide storage and neuropathological abnormalities, but, despite these significant corrections, the treated mice continue to develop neuromotor disability (Sevin et al., 2007b). The results of this study emphasize the concept that treatment for MLD has to be initiated at the earliest age possible.

In a followup study, the safety and efficacy of direct CNS administration of an AAV5 vector expressing the *ARSA* gene were assessed in a nonhuman primate (NHP; *Macaca fascicularis*; Colle et al., 2010). The number and location of vector administration sites were chosen to maximize the breadth of coverage in the CNS to facilitate translation to human clinical evaluation. The administrations were either into three selected areas of the centrum semiovale white matter or into the deep gray matter nuclei (caudate nucleus, putamen, and thalamus) of six NHPs. AAV5 vector expressing *ARSA* was administered at two doses (3.8×10^{11} or 1.9×10^{12} genome copies). The administration sites differed from those previously used in mice to evaluate whether vector injections into the white matter could be dispersed in an anisotropic manner along neuronal tracts to transduce distant neurons in the white matter tracts (Krauze et al., 2005; Hadaczek et al., 2009). Additional target sites in the striatum and thalamus were targeted in a single NHP in an effort to test whether axonal transport and distribution through well-connected afferent regions could amplify the spread of AAV vectors and/or ARSA enzyme into distal regions of the CNS. Vector distribution and expression and activity of human ARSA were evaluated. The procedure was well tolerated, without any signs of adverse effects. There were no significant changes in neurobehavioral examination postvector administration. AAV vector was detected in a brain volume that corresponded to 37–46% of the injected hemisphere. ARSA enzyme was expressed in multiple interconnected brain areas, and ARSA activity was increased by 12–38% in a brain volume that corresponded to 50–65% of the injected hemisphere. Importantly, this was above background endogenous levels, so the increased ARSA detected was much higher than that required for therapeutic benefit (5–10%). These data provide evidence of expression and spread of ARSA that could translate into potential benefits of CNS gene therapy in patients with MLD.

AAVrh.10-Mediated Gene Therapy for CLN2

Prior to the discussion of the use of AAVrh.10 serotype for treatment of MLD, we briefly outline our

experience with AAV gene therapy treatment of another CNS disorder, LINCL, to highlight the lessons learned in selection of AAV serotypes for long-term efficacy and safety. Our group has had a longstanding program in the development of gene therapy for LINCL (CLN2 disease), a lysosomal storage disorder caused by mutations in the *CLN2* gene and the resulting deficiency of tripeptidyl peptidase I (TPP-I). In studies with AAV serotype 2- or 5-mediated transfer of the *CLN2* cDNA (1.2×10^9 genome copies [gc] intraparenchymally) to the CNS of 6-week-old *CLN2*^{-/-} mice, we demonstrated expression of the gene product TPP-I and clearance of CNS storage granules, but there was no improvement in the disease phenotypes or survival (Passini et al., 2006). This was followed by studies in wild-type rats, in which AAV serotypes 2, 5, 8, rh.10, each with an identical *CLN2* expression cassette, were compared (2.5×10^9 gc/serotype/rat, left striatum; Sondhi et al., 2007). AAVrh.10 provided the highest TPP-I level and maximal spread beyond the site of injection (Sondhi et al., 2007). The AAVrh.10-based vector functioned equally well in naive rats and in rats previously immunized against human serotypes of AAV (Sondhi et al., 2007). Next, we demonstrated that administration of AAVrh.10hCLN2 (1.6×10^{11} gc) bilaterally in four locations per hemisphere of the CNS of *CLN2*^{-/-} mice (7 weeks old, mildly symptomatic) led to widespread TPP-I activity greater than 1-log levels compared with that seen in wild-type mice (Sondhi et al., 2007). Importantly, the AAVrh.10hCLN2-treated *CLN2*^{-/-} mice had significant reduction in CNS storage granules and demonstrated improvement in gait, nest-making abilities, seizures, balance beam function, and grip strength as well as having a survival advantage (Sondhi et al., 2007). Based on the success of these initial studies, we assessed the hypothesis that early administration of AAVrh.10hCLN2 would impact therapeutic outcome. To test this, AAVrh.10hCLN2 vector was administered to the CNS of *CLN2*^{-/-} mice at 2 days (1.1×10^{11} gc at three sites per hemisphere) or at 3 or 7 weeks of age (1.6×10^{11} gc, divided equally among four locations in each hemisphere; Sondhi et al., 2008). Although all treatment groups showed a significant increase (>1-log) in total TPP-I activity over wild-type mice, mice treated as neonates had higher levels of TPP-I activity throughout the brain, even at 1 year after administration. Using behavioral markers, the 2-day-treated mice demonstrated a marked improvement over mice treated at 3 or 7 weeks or over the untreated mice. Finally, neonatal administration of AAVrh.10hCLN2 was associated with a large improvement in survival, with a median time of death of 376 days, whereas it was 277 days for 3-week-old treated mice, 168 days for 7-week-old treated mice, and 121 days for untreated mice (3.1-fold, 2.3-fold, and 1.4-fold, respectively, greater survival than the untreated *CLN2*^{-/-} mice; Sondhi et al., 2008). The conclusion from this study is that early detection and treatment are essential for maximal benefits of gene therapy which likely is also applicable to other lysosomal storage diseases such as MLD.

We next evaluated the safety of direct CNS administration of AAVrh.10hCLN2 for a potential clinical study for the treatment of CLN2 disease (Sondhi et al., 2012). Rats and NHPs administered doses that were scalable to humans and delivered to clinically relevant sites had an acceptable safety profile in addition to mediating an acceptable level of CLN2 expression in the CNS. A dose of 10^{11} gc was administered bilaterally to the striatum of rats with sacrifice at 7 (to assess acute effects) and 90 (to assess longer-term effects) days. There were no significant adverse effects except for mild vector-related histopathological changes at the site of vector administration. Next, AAVrh.10hCLN2 was administered to the CNS of eight NHPs (African green monkeys, total dose of 1.8×10^{12} gc). Postvector administration, these animals did not differ from controls in any safety parameter except for mild to moderate white matter edema and inflammation localized to the administration sites of the vector. There were no clinical sequelae (in-life assessment of general safety parameters, complete blood count, serum chemistries, and detailed behavioral testing) to these localized histopathologic findings. TPP-I activity was >2 standard deviations over normal background levels in >30% of the brain of these monkeys at 90 days postvector administration (Sondhi et al., 2012). Together these findings establish the dose and safety profile for human clinical studies for the treatment of LINCL with AAVrh.10hCLN2 and lay the groundwork for a clinical study (NCT01414985, NCT01161576; ClinicalTrials.gov) that is currently ongoing and also for the use of this AAV serotype for treatment of other such diseases, such as MLD.

AAVrh.10-Mediated Gene Therapy for MLD

Based on the successful use of AAVrh.10-mediated gene transfer for the treatment of the mouse model of LINCL, studies were carried out to evaluate the impact of AAVrh.10 vector expressing the *ARSA* gene (AAVrh.10-hARSA) in the MLD mouse model in direct comparison with the AAV5 vector (Piguet et al., 2012). In the Piguet et al. study, both AAV serotype vectors were administered to MLD mice at 2.3×10^6 vg via an intraparenchymal route to the right striatum. The primary findings were that 1) AAVrh.10 transduced more neurons both at sites proximal to administration site and distal brain loci than AAV5, 2) AAVrh.10 expressing GFP (a nonsecreted protein) was used as a control to demonstrate that the secreted ARSA mediates significant retro- and anterograde transport of ARSA protein expression beyond the GFP-labeled cells, 3) there were AAVrh.10-mediated greater ARSA levels and broader spread of vector and ARSA activity in the brain than with AAV5, and 4) AAVrh.10hARSA mediated correction of sulfatide accumulation in oligodendrocytes, even at a very advanced stage of disease. These results strongly supported the use of AAVrh.10hARSA vector for CNS gene therapy in rapidly progressing early-onset forms of MLD.

As a next step toward clinical translation, an NHP study was carried out focused on establishing the best

method of vector delivery to obtain the maximal distribution of transgene product. We compared the vector administration protocol for the LINCL clinical study (Worgall et al., 2008; Souweidane et al., 2010) with four alternate protocols focusing on maximal ARSA distribution evaluated by morphological and molecular methods, including 1) delivery to deep gray matter with overlaying white matter deposits, 2) deep gray matter with convection-enhanced delivery, 3) intraventricular delivery, and 4) intra-arterial delivery with mannitol-mediated opening of the blood–brain barrier (Rosenberg et al., 2014). In each mode of delivery, a total dose of 1.5×10^{12} gc of AAVrh.10hARSA-FLAG (the vector was tagged with the FLAG epitope to aid in detection of vector-derived human ARSA against the wild-type monkey ARSA background) was delivered. After 13 weeks, the distribution of ARSA activity was assessed in the NHPs by measuring the levels of functional ARSA activity in the CNS by subsectioning the brain into 1-cm³ cubes. ARSA activity in each of the three direct intraparenchymal administration routes (white matter and gray matter) was significantly higher than in controls that received PBS, but administration by the intraventricular and intra-arterial routes failed to demonstrate measurable levels above control. These enzymatic assay results were corroborated by immunohistochemical analysis. Among the five routes studied, administration to the white matter alone generated the broadest distribution of ARSA across the CNS, with 80% of the brain displaying more than a therapeutic (10%) increase in ARSA activity above that of PBS controls. We did not observe any significant toxicity with any delivery route (in-life assessment of general safety parameters, complete blood count, serum chemistries, and detailed behavioral testing), other than inflammatory changes that were seen by histopathology at or close to sites of administration. From these findings we concluded that AAVrh.10-mediated delivery of ARSA, delivered in the same fashion as AAVrh.10hCLN2, is safe, yields wide distribution of ARSA, and is a suitable route of vector delivery.

Two toxicology studies were carried out in NHPs to assess the safety of direct administration of AAVrh.10hARSA into the CNS. The results of the first study have been published and are summarized below, and a second study was recently completed, with data being analyzed for a final report and publication. In the first study, NHPs received either 1.1×10^{11} gc/hemisphere (unilateral or bilateral injection) or a 5-fold higher dose (5.5×10^{11} gc/hemisphere, bilateral injection; Zerah et al., 2015). NHPs were subjected to various in-life assessments, including MRI brain imaging, and were sacrificed at 7 or 90 days after administration to assess acute and longer-term effects. No evidence of toxicity was observed in any clinical or biological parameters. In addition, no treatment-related adverse findings were seen in any of the organs assessed. In NHPs that received the 5-fold higher dose, MRI T2 hypersignals suggested a region of edema that correlated with histopathological findings observed in the brain 90 days after administration. This finding was mini-

mal or completely absent in the MRI scans of the lower-dose cohort. After administration of the lower dose, AAVrh.10hARSA was detected in a large part of the injected hemisphere and ARSA activity exceeded the normal endogenous activity level by 14–31%. From these data, we conclude that the human dose equivalent to the lower dose (1.1×10^{11} gc/hemisphere) will likely be safe when tested in the human setting. We recently completed another toxicology study in NHPs, which assessed two additional doses of AAVrh.10hARSA (2.85×10^{10} and 1.5×10^{12} gc/brain), bracketing the lower dose tested in our study described above, and included two controls (PBS and an AAVrh.10Null vector, which has an expression cassette with a nontranslatable sequence, to act as a control for the vector). The results of this study are being analyzed, but the preliminary data are consistent with the prior study.

LESSONS LEARNED AND PATH FORWARD

Among the gene transfer viral vectors, AAVs have a promising clinical track record with regard to both safety and efficacy. There is a growing list of neurological disorders currently being treated with AAV vectors in clinical trials, including Canavan disease, late-infantile neuronal ceroid lipofuscinosis, MPS IIIA, Alzheimer's disease, and Parkinson's disease (Janson et al., 2002; Tuszyński and Blesch, 2004; McPhee et al., 2006; Kaplitt et al., 2007; Worgall et al., 2008; Mandel, 2010; Souweidane et al., 2010; Kells et al., 2012; Tardieu et al., 2014). Our data and those of others has shown that CNS-targeted AAV gene therapy vectors have become a promising modality for treating leukodystrophies. The strategy to deliver the therapeutic gene directly to the CNS offers a permanent, lifelong correction of the mutated/missing enzymes. In contrast to ERT, gene therapy applied directly to the CNS is not limited by the blood–brain barrier or by the need for repeated lifelong treatment because of the half-life of the therapeutic protein. It does not have the risk of myeloablative chemotherapy or graft rejection as with BMT and ex vivo HSC transplantation.

With this rationale, a phase 1/2, open-labeled, monocentric clinical trial (NCT01801709; ClinicalTrials.gov) has begun in France to assess the safety and efficacy of AAVrh.10hARSA for treatment of early-onset forms of MLD by gene therapy (Aubourg, 2016). Similar to our current clinical trial for CLN2 disease (LINCL), the MLD trial in France is using direct CNS administration of AAVrh.10hARSA at 12 sites, through six image-guided tracks with two deposits per track, into the white matter of both brain hemispheres. A low dose (1×10^{12} gc) was administered to the first two patients, whereas the last three received a 4-fold higher dose (4×10^{12} gc). Safety and efficacy are being evaluated based on clinical, neuropsychological, radiological, electrophysiological, and biological parameters. We are also planning a submission of an investigational new drug application for initiating testing of this therapy for MLD in the United States.

As we have outlined in this Review, the findings from the CLN2 and MLD studies have direct applications for other LDs, including globoid cell leukodystrophy (GCL, Krabbe's disease). Similarly to MLD, HSCT has been shown to be efficacious in Krabbe's disease but only in patients with later onset of disease or in presymptomatic infants (Krivit et al., 1999b; Helman et al., 2015). Similarly, in studies of UCBT in infants with infantile Krabbe's disease, there is a marked difference in survival rates and neurologic outcomes when UCBT is performed in younger asymptomatic children/infants (Escobar, 2005). Rafi et al. (2005) have demonstrated that Krabbe's disease can be successfully treated in animal models by AAV-mediated gene therapy. As shown in their earlier studies, administration of AAV1 serotype encoding the mouse *GALC* gene (AAV1-mGALC, 3×10^{10} viral particles, intracerebroventricularly and intraparenchymally) in neonatal "twitcher" mice (a naturally occurring mouse GCL defect) resulted in dramatic improvements in neurological condition of the affected mice and led to an increase in *GALC* activity across the CNS. In a more recent study, based on the success of AAVrh.10 for the treatment of CNS-related manifestations of various neurodegenerative diseases, Rafi et al. (2012) treated 2-day-old neonatal twitcher mice with the AAVrh.10 serotype encoding the mouse *GALC* gene (AAVrh.10-mGALC, 7.6×10^9 viral particles) via three separate and combined routes, intracerebroventricular, intravenous, and intraparenchymal routes. They found that survival was maximized when the AAV vectors were administered in a combination of all three routes. The use of AAVrh.10 and other AAV serotypes for the treatment of Krabbe's disease may pave the way forward for new clinical trials and successful long-term treatment modalities for patients afflicted with this LD or other LDs. Toxicology studies have already demonstrated that direct administration of AAVrh.10 into the CNS is safe and efficacious, and clinical trials are ongoing for other indications, which should allow a faster translation of AAVrh.10 gene therapy for Krabbe's disease to clinical use.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all the information required to draft the Review and were involved in establishing the outline of how to describe the clinical development of a gene therapy strategy for the treatment of leukodystrophies. Drafting of the manuscript: JBR, SMK, DS. Critical revision of the manuscript for important intellectual content: DS, RGC, PA.

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