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Recombinant adeno-associated virus vectors in the treatment of rare diseases

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Abstract

Introduction—An estimated 25 million Americans are living with rare diseases. Adeno-associated virus (AAV)-mediated gene therapy is an emerging therapeutic option for the more than 7,000 identified rare diseases. This paper highlights the benefits of AAV therapy compared to conventional small molecules, discusses current pre-clinical and clinical applications of AAV-mediated gene therapy, and offers insights into cutting edge research that will shape the future of AAV for broad therapeutic use.

Areas covered—In this review the biology of AAV and our ability to generate disease-specific variants is summarized. Limitations of current therapy are reviewed, with an emphasis on immune detection of virus, viral tropism and tissue targeting, and limitations of gene expression. Information for this review was found using PubMed and clinicaltrials.gov.

Expert opinion—Currently the scope of clinical trials of AAV gene therapy is concentrated in an array of phase I/II safety trials with less than two dozen rare diseases featured. Pre-clinical, translational studies are expanding in number as developments within the last decade have made generation of improved AAV vectors available to more researchers. Further, one bottleneck that is being overcome is the availability of disease models, which will allow for improved preclinical testing and advancement of AAV to more clinical applications.

Keywords

Adeno-associated virus; AAV vectors; AAV; Gene therapy; Orphan disease; Rare disease

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1. Introduction to AAV-mediate gene therapy

The Orphan Drug Act of 1983 defined rare diseases as those affecting less than 200,000 Americans. However, with more than 7,000 monogenic rare disease listed by the National Genetic and Rare Disease Information Center (GARD), more than 25 million Americans are affected illustrating the significance of these collective disorders. Historically pharmacological small molecule treatments have been the only option for managing a few rare diseases, but these efforts have a history of off target effects, need to be taken indefinitely, or have reduced efficacy over time as a result of dose tolerance. Protein therapy has filled a number of niches and continues to look promising. However with the advent of gene transfer technology, new options are on the horizon. Emerging as a promising therapeutic, adeno-associated virus (AAV) vectors provide a convenient packaging system to deliver disease-specific, long-term therapies based on virus-mediated delivery of corrected genes or endogenous gene knockdown to abrogate disease phenotypes. To date approximately 50% of the genes responsible for rare diseases have been identified, with genotyping being the limiting factor¹. However, next-generation sequencing is quickly identifying target genes and it is expected that the genetic mutations causing most rare diseases will be known in the next decade¹. This review is organized to first discuss the biology of AAV and current strategies to improve AAV gene therapy vectors, while the second half will discuss individual diseases and current AAV research, focusing on pre-clinical studies and clinical trials (Table 1).

2.1 Biology of AAV

AAV is a non-autonomous single-stranded (ss) mammalian DNA virus (family *Parvoviridae*, genus *Dependovirus*) that requires helper functions, usually provided by associated viruses (such as adenovirus) to complete its life cycle. Thirteen major human and non-human primate (NHP) serotypes have been identified, with more than 100 serotypes identified from NHPs and several other species. The approximate 4.7kb AAV genome is flanked by 145 nucleotide inverted terminal repeats (ITRs) and contains three identified open reading frames (ORFs) encoding eight proteins required for virus propagation². At 25nm, the mature AAV virion consists only of capsid proteins and a ssDNA genome, with both positive and negative DNA strands being incorporated equally². Although, AAV is ubiquitous in nature and it is estimated that more than 70% of humans are seropositive for one or more serotypes, AAV has not been directly implicated in human or animal diseases^{3, 4}.

More than 30 years ago it was demonstrated that the ORFs of AAV could be supplied in trans allowing the replication and capsid packaging of transgenic DNA flanked by the AAV ITR sequence (termed recombinant AAV or rAAV). In conjunction, with a helper virus free production system, rAAV has been exploited in the basic sciences and as a therapeutic drug in >100 clinical trials for diverse diseases⁵. Most important for safety concerns and therapeutic applications, all recombinant AAV (rAAV) replication, capsid, and packaging protein sequences are replaced with expression cassettes encoding the therapeutic transgene of interest. This allows for generation of replication defective recombinant AAV (rAAV) vectors that have limited antigenic properties and low risk of host cell genome integration.

Significantly, production of research-grade rAAV has been optimized with a scalable format that achieves yields of purified rAAV of 10^{13} to 10^{15} DNase-resistant particles (DRP)/ml⁶⁻⁸.

2.2 rAAV vector development strategies

The ability of rAAV to deliver therapeutic genes for correction of rare disease phenotypes makes it an exciting vector for clinical use. Challenges for rAAV therapies are being addressed in current research, including: 1) evasion of immune detection (for both the vector capsid and transgene product), 2) manipulating rAAV tissue tropism, and 3) enhancing the transgenic expression cassettes. These research areas aim to overcome premature clearance/immune response to treatment, restrictive targeting of rAAV to specific tissue types and/or to improve transduction of cells that are currently refractory, and to optimize therapeutic gene expression for increased efficacy at low vector doses in clinical applications.

2.3 Evasion of rAAV immune detection

Pre-existing as well as adaptive immunity against AAV is a significant challenge to current therapies and has been reviewed in detail⁹⁻¹². Briefly, development of AAV capsid specific memory B and T cells occurs in childhood in much of the population¹³. Early response to AAV results in production of neutralizing antibodies (NAbs) and memory immune cells that can prevent later rAAV transduction and eliminate transduced cells, respectively. Clinical trials have offered many insights into the humoral responses to rAAV in addition to cell-mediated clearance of transduced cells.

Understanding the NAb response to rAAV is especially important as therapeutic efficacy may depend on the treatment of rare diseases shortly after birth or in early childhood. There does appear to be an optimal treatment window during the first year following birth where maternal NAbs diminish prior to self-made NAbs increasing to a plateau in adolescence^{3, 14}. However, even with delivery of high concentrations of rAAV particles, clearance of virus by NAbs can greatly reduce therapeutic efficacy by preventing transduction and limiting long-term gene expression¹⁵. It should be noted that the prevalence of NAbs has not been examined for all AAV serotypes. One comprehensive study examined NAbs against some of the most common serotypes used for gene therapy, rAAV1, 2, 7, and 8, focusing on prevalence worldwide⁴. The highest prevalence of NAbs was against AAV2, followed by AAV1, with lower NAb prevalence detected for AAV 7 and 8⁴. Interestingly, another study with nonhuman primates (NHPs) suggests that even non-NAbs can result in premature clearance of AAV8 and that therapy is best in NHPs without pre-existing immunity¹⁶—unfortunately in humans, this is only 30% of the population.

As rAAV vectors encode no viral genes, the humoral and cell-mediated immune responses are directed against the vector capsid as well as expressed transgenes. Perhaps the most obvious solution would be co-therapy with immunosuppressive drugs, however this will not remove already circulating NAbs, may require extended application, and may not be applicable in rare disease patients with weakened immunity. Further, NAb depletion may result in the removal of antibodies that are cross-reactive for other pathogens, which also could be potentially harmful to immunosuppressed patients. Consequently, other methods of immune evasion are being researched.

Different rAAV serotypes are being explored for gene therapy use, but capsid homology may limit this approach. Antibody cross reactivity was seen against rhesus monkey derived AAVrh10 compared to human serotypes AAV9 and 2 in mice ¹⁷. In another study, AAV2 NAbS were cross-reactive for AAV 5 and 8, and limited the alternative serotype approach in pediatric patients with hemophilia ¹⁸. One group demonstrated the effectiveness of goat-derived rAAV with 94% homology to human AAV5 that was able to evade NAbS, with epitope difference predicted at the capsid surface or spike-like protrusions ¹⁹.

Current research is developing additional NAb-resistant rAAVs mainly through capsid protein modification. Directed evolution using random mutagenesis generates capsid protein sequence libraries that require screening for NAb-resistance ²⁰. More specific to the antigenic regions of the capsid proteins, site-directed mutagenesis is being explored to select for rAAV with reduced NAb binding ²¹⁻²³. Further, the use of bioinformatics is allowing for generation of high-resolution maps of sequences required for structure versus NAb recognition sites that may lead to rational design of Nab resistant capsids ²⁴. In addition to genetic modification, chemical immunoshielding of rAAV capsids with polyethylene glycol or immunoshielding with natural extracellular vesicles has been attempted, with the later showing 4000-fold increase in transduction efficiency ²⁵⁻²⁷. Even more, aptamer immunoshielding has shown promise for other viruses and may improve rAAV evasion of NAbS ²⁸.

Following rAAV evasion of NAbS is a need to understand cell-mediated clearance of transduced cells. In fact, prior to early clinical trials, animal models failed to predict CD8⁺ T-cell as a limiting factor in rAAV therapy, possibly occurring because of pre-existing immunity to AAV ²⁹. Proteasomal processing of vector capsids or expressed transgenes results in antigen presentation and recognition by cytotoxic T cells. As rAAV is non-replicating, detection of capsid antigen in transduced cells should be limited following treatment. Surprisingly, depending on the vector or target tissue, capsid antigen was detectable weeks to years post treatment ^{29, 30}. Here again, site-directed mutagenesis of the capsid has been attempted to evade the cell-mediated responses. Specific tyrosine residues were replaced to prevent ubiquitination, proteasomal degradation, and subsequent major histocompatibility complex (MHC) presentation ³¹. Regarding expressed transgenes, like the capsid, proteasomal degradation is followed by MHC presentation. However, many factors may influence the cell-mediated response. Of note from numerous clinical observations is prior exposure the therapeutic protein, the route of administration, the target tissue, and even the disease state of the organ, as reviewed previously ¹². Important for clinical use, a direct correlation between dosage and cell-mediated immunity has been observed. Interestingly, the kinetics of T cell activation and clearance of transduced cells may be serotype dependent ^{29, 32}.

Less explored is a role for cellular innate immunity and its effect on adaptive immunity against rAAV. The cytosolic sensor, toll-like receptor 9 (TLR9), identifies the rAAV genome and promotes type I interferon antiviral signaling and inflammation that may enhance the adaptive immune response against rAAV ^{33, 34}. Additionally, as TLR9 detects CpG sequences, depletion of CpG ligands from the rAAV genome reduces adaptive immune responses and improves transduction ³⁵. Deficiency in MyD88, an effector molecule of

TLR9, results in decreased NAb responses to rAAV³⁶. Though it is unclear if other cellular proteins promote immune responses like TLR9, promyelocytic leukemia protein (PML) or the DNA damage complex Mre11/Rad50/Nbs1 (MRN) were shown to inhibit rAAV second strand synthesis and/or inhibit gene expression independent of rAAV DNA synthesis through an unknown mechanism³⁷⁻³⁹. These studies highlight the need for continued investigation of cellular host anti-viral responses and a link to humoral and cell-mediated immunity against rAAVs.

2.4 rAAV capsid modification for targeted tropism

Cell surface attachment of rAAV is serotype dependent and numerous cell surface receptors have been identified. In general, cell surface receptors like heparin sulfate proteoglycan⁴⁰, O- and N-linked sialic acids^{41, 42}, galactose⁴³, and ganglioside GM1⁴⁴ contribute to rAAV serotype tropism. Mutagenesis of rAAV capsids allows for attachment to other cell receptors, like chondroitin sulfate⁴⁵ and $\alpha v\beta 8$ integrin⁴⁶, which may help with targeting refractory cells. Importantly, rAAV must be able to infect and transduce target cells where therapy is required. Depending on the rare disease, the therapeutic need could be localized or systemic. The ability to generate designer rAAVs that home to specific tissue types has been describe in numerous reviews about gene therapy of the central nervous system (CNS)⁴⁷⁻⁵⁰, eye^{51, 52}, heart^{53, 54}, lungs^{55, 56}, ear⁵⁷, liver⁵⁸, bones and joints⁵⁹, muscle^{60, 61}, or adipose^{62, 63} tissue. Many of the ongoing clinical trials of rAAV are exploring rare diseases that require tissue specific treatment (Table 1).

Direct targeting involves inserting small peptide or ligand sequences into the capsid sequence⁴⁵. This approach has improved targeting to tissues including muscle²⁴ or lungs³⁹, but is limited in that it may inhibit binding of the rAAV to natural cell surface receptors or generate new epitopes for immune response. Another approach involves using capsid sequences from different serotypes to generate mosaic or chimeric capsid protein libraries. These libraries can be used to produce rAAVs with tropism different from the parental vector and can be easily screened to determine tissue specificity and examine transduction efficiency^{64, 65}.

Indirect targeting has also being explored. Here, a mediator molecule interacts with the rAAV vector and a specific cell receptor. Examples include bispecific antibodies⁶⁶ or biotin^{67, 68}. Additionally, chemically directed tropism can be achieved using chemicals that block natural rAAV receptors or prevent virus capsid ubiquitination and are reviewed for rAAV2⁶⁹. These approaches allow for targeting without modification to the capsid, which may inhibit transduction efficiency.

2.5 Gene expression from rAAV vectors

While the previous two sections focus on steps that are critical for the refinement of future rAAV application, long-term gene expression is the most important aspect of gene therapy vector. The limited packaging size of rAAV vectors is a major limitation when considering gene length options and has been reviewed⁷⁰. Additionally, the rAAV ITR sequences (which have natural promoter activity or contain cellular transcription factor binding sites) can influence transduction efficacy and gene expression levels. Different methods are being

examined to improve rAAV packaging size as well as identify promoter and cellular transcription factor binding sequences within the rAAV genome.

Most therapies are based on single stranded rAAV genomes where a transgene cassette is placed between 2 ITR sequences, but other vectors exist. For instance, the self-complementary AAV (scAAVs) genome was intentionally produced based on a replication intermediate that allows self-annealing of the ssDNA, thus producing a duplex molecule capable of transcription⁷¹. When tested, scAAV mediates faster and more robust transgene expression. Unlike ssAAV, host mediated second-strand synthesis of the scAAV genome is not required for transgene production. However, due to the requirement for self-annealing of scAAV genomes, scAAV-based cassettes need to be less than half of the approximate 5kb capsid capacity restricting their use to the treatment of diseases requiring large DNA delivery⁷². This method is useful for smaller transgenes and can be applied to rare diseases that require knockdown of mutant genes through shRNA, miRNA, or similar methods.

Expression of larger transgene cassettes is possible using several genetic strategies relying on host-mediated reconstruction of the larger, desired, cassette. Currently, rationally designed overlapping vectors and several types of concatemerization dependent vectors have been described which mediate large gene transduction. In addition, the attempted packaging of cassettes >5kb results in encapsidated rAAV genome fragments (termed fragment AAV or fAAV) that are reconstructed into the intended larger cassette. In the literature, the efficiency of these different approaches is inconsistent, although, it is generally agreed upon that transduction efficiency is substantially decreased when compared to intact ssAAV. No AAV large gene delivery strategies have been approved for clinical application. Importantly, as the efficiency of transgenic DNA reconstruction likely correlates to tissue type and cell cycle status, further investigation is needed to refine AAV large gene delivery. Furthermore, as host replication and DNA repair machinery may be affected in some rare diseases, particular large gene strategies may prove ineffective.

Focusing on current vectors, it is not surprising that transduction efficacy and gene expression can be influenced at the rAAV genome level. Numerous promoters of constitutive gene expression have been studied and compared for rAAV transgene expression: human β -actin, human elongation factor-1 α , a chicken β -actin variant, cytomegalovirus (CMV), simian virus 40, and herpes simplex virus thymidine kinase¹⁹. While this approach is excellent for systemic delivery and expression, in rare disease that may require targeted expression, tissue specific promoters are being explored. In just one of many examples, the use of liver specific promoters can influence gene expression profiles⁷³. Here bioinformatics was utilized to identify cis-acting regulatory modules (CRMs) that enhance liver specific gene expression 10 to 100-fold⁷⁴.

Interestingly the rAAV genome flanking ITR sequences have very low promoter activity⁷⁵. Even more, the ITRs contain sequences that may act as binding sites for host suppressor proteins. In one instance, a 20-nucleotide ITR sequence that contained homology to a NF- κ B-repressing factor binding site was substituted with a sequence for transcription factor binding and resulted in enhanced transduction⁷⁶. Future identification of other regulatory

sequences in the rAAV genome, ITR or otherwise, will allow for rational design of promoters or sequence modification for disease specific application.

3. Rare disease and current rAAV pre-clinical and clinical studies

Gene therapy has been explored for more than 40 years, but to date only one rAAV vector, Glybera, for treatment of the rare disease lipoprotein lipase deficiency (LPL), is approved for use in the European Union. Following a rocky start to clinical trials in 1999, gene therapy is finding its stride and currently rAAVs account for 5.6% of the approximate 2076 gene therapy trials (<http://www.abedia.com/wiley/vectors.php>)⁷⁷. The list of rare diseases being studied in clinical trials with rAAV therapies in table 1 is promising, with several even in phase III trials. Additionally, rare diseases currently being addressed by other gene therapy vectors may be treatable with rAAV. Combined data from preclinical and clinical investigations are generating an abundance of information to guide researchers in choosing optimal vector-disease combinations. It is clear that the rAAV serotype as well as the dose and route of administration impact therapeutic outcomes. Current clinical trials (Table 1) inject rAAV via many routes: intravenous, intramuscular, intrapleural, into specific brain regions during surgery, subretinal, intranasally, intrahepatic, peripheral vein infusion, or convection-enhanced delivery (CED) to the putamen, intravitreal, or intracerebral. The vectors most often used are based on the most characterized serotype AAV2, but vectors designed from rAAV1, rAAV8, rAAV9, and rAAVrh.10 have also entered the clinic due to broader or enhanced transduction. Results of preclinical and clinical trials offer insights into safety, immune reactions, disease response to treatment, and length of therapeutic efficacy.

An abundance of reviews describe rAAV rare disease gene therapy, including psychiatric disorders, neurodevelopmental disorders, lysosomal storage diseases (LSDs), amyotrophic lateral sclerosis, glycogen storage diseases, inborn metabolism errors, Duchenne muscular dystrophy, and epilepsy to name a few of the most recent in the literature. Several major approaches for using rAAV-mediated gene therapy of rare diseases are being explored: 1) expression of wild type proteins to correct for mutant genes, 2) expression of smaller, functional variants of wild type proteins not amenable to the restrictions of rAAV packaging, or 3) gene silencing through expression of small RNAs. Due to the recent explosion of rAAV research and rare disease, this review groups the rare disease below and highlights important historic milestones as well as the most recent findings in pre-clinical research and clinical trials.

3.1 Retinal diseases

3.1.1 Choroideremia (CHM)—Mainly affecting males, X-linked CHM results in loss of photoreceptors, retinal pigment, and choroidal vessels and leads to blindness by middle age. Because rAAV8 can be used in a ten-fold lower dose than rAAV2 and transgene expression reaches maximal levels sooner⁷⁸, the authors explored rAAV8 encoding the Rab escort protein 1 (REP1), demonstrating safety and significant stalling of degeneration and long term rescue of retinal-cortical function in $Chm^{null/WT}$ mice⁷⁹. Optimistic results with gain of visual acuity came from a phase I/II trial with rAAV2 encoding the REP1 protein for

treatment of choroideremia. Patients administered subretinal rAAV2 exhibited improved rod and cone function with a direct correlation of dosage to improved retinal sensitivity ⁸⁰.

3.1.2 Leber congenital amaurosis (LCA)—This group of heritable retinal dystrophies is characterized by loss of visual function in childhood caused by mutations of more than 15 known genes. Significantly, following reversal of blindness in animal models, clinical trial results have demonstrated long-term safety and markedly increased visual sensitivity in multiple patients with LCA2, caused by retinal pigment epithelium-specific-65-kDa (RPE65) deficiency ⁸¹. Of the current ongoing rare disease clinical trials, Rep65 rAAVs make up the largest cohort, with 10 studies in phase I, II, or III. Additionally, in preclinical studies of LCA12, administration of scAAV8 Y733F capsid mutant expressing retinal degeneration protein 3 (RD3) from a photoreceptor-specific promoter results in photoreceptor cell survival. Rd3 expression from the scAAV8 vector is seen 1 week following treatment in mice, compared to 4 to 6 weeks for previously studied rAAV5 ^{82, 83}.

3.1.3 Retinitis pigmentosa (RP)—RP is a general term for a group of inherited diseases that exist as autosomal-recessive, autosomal-dominant, or X-linked diseases, with multiple causative genes. In a rare autosomal-recessive form of RP, mutation of the human receptor tyrosine kinase MER (MERTK) gene causes a loss of photoreceptors. Subretinal injection of AAV2-CMV-MerK into Royal College of Surgeons (RCS) rats resulted in a 2.5-fold higher number of functional photoreceptors compared to controls up to 9 weeks post treatment ⁸⁴. Similar results were seen in treatment of an autosomal dominant RP where disease arises from mutation to the rhodopsin (RHO) gene. RHO augmentation using rAAV2/5 in Rho^{-/-} mice preserves the survival of rod cells ⁸⁵. Addressing a dominant RP cone-rod dystrophy caused by mutated guanylate cyclase-activating protein 1 (GCAP1), scAAV2/8 encoding allele specific shRNA against GCAP1 was injected subretinally in mouse models. Expression of the shRNA was strong 1 week post injection and gene silencing lasted 1 year with treatment enhancing photoreceptor survival and delaying onset of degeneration ⁸⁶. Lastly, a common X-linked form of RP (XLRP) is caused by mutation to the RP GTPase regulator (RPGR) gene. No treatment is available to date, but in two canine models rAAV2/5-mediated expression of RPGR in rods and cones rescues photoreceptor blindness and prevents disease onset at an early age ⁸⁷.

3.1.4 Age related macular degeneration (AMD)—Unlike the retinal diseases above, there is no causative mutation known for AMD. However, rAAV therapy may still prove effective. Here, localized inflammation induced by interleukin (IL)-17 may be retinotoxic ⁸⁸. Use of rAAV2 encoding the soluble IL-17 receptor prevented retinopathy in mice and fewer lesions and reduced photoreceptor atrophy were observed ⁸⁸. One phase I clinical study is ongoing and is based on rAAV2 expressing a chimeric soluble Fms-Related Tyrosine Kinase 1 (Flt1) receptor that suppresses the proangiogenic vascular endothelial growth factor (VEGF). Preclinical data with intravitreal injection of the same vector in mice, rats, and monkeys demonstrated inhibition of pathological neovascularization as well as tolerated therapy and long-term gene expression ⁸⁹.

3.2 Nervous system related diseases

3.2.1 Spinal muscular atrophy (SMA)—At least four different mutated genes are known to cause neuron degeneration, progressive paralysis, and childhood death in SMA. When expressed at low levels, the survival of motor neuron 1 (SMN1) protein is a causative agent and has been studied in rAAV therapies. Delivery of scAAV9 encoding SMN1 to mice and NHPs results in widespread transgene expression in spinal cord motor neurons and complete rescue of SMA phenotype in mice. Even more, the study noted that a ten-fold lower dose was required for the same expression profile in NHPs when delivered to cerebral spinal fluid compared to intravenous injection⁹⁰. It has also been noted that phosphatase and tensin homolog (PTEN) protein depletion leads to increased neuron survival in SMA. Treatment of mice with rAAV6 encoding shRNA against PTEN or scAAV9 encoding siPTEN resulted in a 3-fold increase in lifespan, suggesting that continued investigation may be beneficial⁹¹. One phase I trial using scAAV9 encoding an enhanced CMV-chicken beta actin hybrid promoter and SMN has not reported any results.

3.2.2 Aromatic L-amino acid decarboxylase (AADC) deficiency—Mutation to the AADC gene impairs biosynthesis of neurotransmitters like serotonin or dopamine that are required for signaling between cells of the central nervous system. In neonatal mice, following intracerebroventricular injection of rAAV9 encoding human AADC, dopamine and serotonin levels rose from 25% and 15% to 100% and 40%, respectively, with improved growth rate and survival as well as partially corrected behavioral abnormalities⁹². Currently one clinical trial is exploring the use of rAAV2 encoding AADC for AADC therapy.

3.2.3 Alternatively, a 2010 phase I clinical trial used rAAV2 encoding AADC for treatment of Parkinson's disease and patients have demonstrated improved motor performance⁹³—Parkinson's can be caused by multiple gene mutations, but progressive loss of AADC occurs in most cases. Another phase I trial with the rAAV2-hAADC-2 vector is ongoing. Additionally, two clinical trials in phase I and I/II are using rAAV2 to express neurotropic growth factors, glial cell derived neurotrophic factor (GDNF) and neurturin (NRTN), respectively, in an effort to support dopamine producing cells. Further as oral therapies L-DOPA may show some benefits, long-term treatment has unwanted side effects. Recently, it was demonstrated that rAAV5 encoding the tyrosine hydroxylase (TH) or GTP cyclohydrolase 1 (GCH1), enzymes that overcome a rate-limiting step in dopamine production, can be used to produce dopamine in Sprague-Dawley rats⁹⁴. Significantly, the group demonstrated that a destabilized dihydrofolate reductase (DD) domain on the N terminus of GCH1 could be used to control expression in a patient specific manner. In this method, addition of activating ligand trimethoprim (TMP), that crosses the blood-brain barrier, prevents GCH1 expression and, in a dose dependent manner, inhibits dopamine production in rAAV-transduced cells⁹⁴.

3.2.4 Fragile X syndrome (FXS)—Mutation to the FMR1 gene encoding the fragile X mental retardation protein (FMRP) results in delayed neurodevelopment and a range of intellectual disability. In the first proof of principle study of rAAV and FXS, intracerebroventricular injection with rAAV9 encoding a major isoform of FMRP improved

neonatal mouse behavior. Physiologically, FMRP expression reached approximately 50% of wild type levels at 56 days post injection ⁹⁵.

3.2.5 Friedreich's ataxia (FRDA)—A mitochondrial disease, FRDA is characterized by neurodegeneration as well as diabetes and hypertrophic cardiomyopathy, with the later being the primary cause of mortality. FRDA is caused by reduced levels of frataxin (FXN), a protein required for synthesis of iron-sulfur clusters. Intravenous application of AAVrh10, a rhesus monkey derived vector, encoding FXN demonstrates high levels of FXN expression in cardiac tissue and prevents onset of cardiac disease in mice ⁹⁶. Significantly, when the vector was used after the onset of heart failure, cardiomyopathy was completely reversed within a few days.

3.2.6 Familial amyloidotic polyneuropathy (FAP)—Mutation of the transthyretin (TTR) gene causes extracellular deposition of amyloid fibrils in the peripheral nervous system and currently, the only treatment is liver transplantation. Treatment of TTR V30M mice with scAAV8 encoding the TTR T119M variant with trans-suppressor activity leads to less destabilized monomers of TTR, more functional TTR tetrameric protein, and reduced non-fibrillar aggregates ⁹⁷.

3.2.7 Huntington disease (HD)—The autosomal dominant HD is caused by expansion of the CAG repeat in exon 1 of the huntingtin (HTT) gene and results in neuron degeneration as a result of increased polyglutamine residues in the Htt protein. The disease is fatal and presents at 35 years or older, with patients living approximately 15 years following diagnosis. Intracranial injection of mice with rAAV2/1 encoding miRNA against the Htt transcript results in more than 80% transduction of striatum cells with significant improvements in behavior and reduction of striatal Htt aggregates ⁹⁸.

3.3 Lysosomal storage diseases

3.3.1 Metachromatic leukodystrophy (MLD)—Accumulation of fats in cells of the nervous system occurs as a result of mutation to the arylsulfatase A or B (ARSA/B) or prosaposin (PSAP) genes. Jugular injection of rAAV9 encoding ARSA and GFP into newborn MLD mice resulted in significant inhibition of accumulation of sulfatide fat in the brain and spinal cord. Importantly, mice had improved balancing abilities, where affected individuals lose motor skills and become unresponsive over time ⁹⁹. Also encoding ARSA, AAVrh.10cuARSA is in a phase I/II clinical trial for MLD. Results in MLD mice demonstrated that within 2 months following intrastriatal injection correction of brain sulfatide storage. Interestingly, axonal transport as well as transduction in neurons and oligodendrocytes was improved compared to rAAV5 encoding ARSA that was shown to alleviate most long-term disease manifestations in mice ¹⁰⁰.

3.3.2 Mucopolysaccharidosis type I (MPS I)—Deficiency in the lysosomal enzyme α -L-iduronidase (IDUA) causes glycosaminoglycans accumulation in tissues and results in neurological disease as well as ocular, skeletal, and cardiac diseases. A MPS I feline model treated with cephalic vein injection of rAAV8 encoding feline IDUA caused enzyme activity at approximately 30% to an excess of normal levels, with the effect lasting for 6 months ¹⁰¹.

In a mouse model of MPS VI, retro-orbital injection of rAAV2/8 encoding thyroxine binding globulin (TBG) fused to the human ARSB gene, as in MLD above, results in improved motor performance ¹⁰². This study compared AAV to conventional enzyme replacement therapy (ERT) and noted similar outcomes and reduced animal stress with rAAV gene therapy.

3.3.3 Pompe disease—Also called Glycogen storage disease type 2, Pompe disease is an LSD characterized by a lack of α -1,4 glucosidase (GAA) and an inability to break down glycogen. Cardiopulmonary failure leads to death in infancy and ERT therapy with GAA has shown improved survival. Cotherapy of rAAV9 or rAAV8 encoding GAA and non-depleting CD4 antibodies suppress anti-GAA responses and results in significant reduction of glycogen accumulation ¹⁰³. In a phase I/II trial for chronic respiratory failure, treatment with rAAV1 encoding α -glucosidase (GAA) results in a 425% increase in periods of unassisted breathing, with no detectable T-cell mediated immune response to the vector ¹⁰⁴.

3.4 Muscle related diseases

3.4.1 Limb-girdle muscular dystrophy (LGMD)—Affecting males and females equally, LGMD results from different recessive, as well as dominant, inheritance patterns. To address the dominant mutation of myotilin (MYOT) in LGMD type 1A, rAAV6 encoding micro RNAs targeting MYOT was administered in the lower limbs of mice. Increased muscle strength and significant functional correction was seen up to 9 months after treatment ¹⁰⁵. In a phase I trial of LGMD type 2D, rAAV1 encoding the alpha-sarcoglycan (α SG) gene with a muscle specific promoter was injected into patient's extensor digitorum brevis (EDB) muscle ¹⁰⁶. Persistent α SG expression was seen for at least 6 months in two out of three patients. Currently an ongoing phase I/II trial is using a scAAVrh74 vector encoding the same promoter-transgene combination for treatment of LGMD type 2C and results have been reported.

3.4.2 Limb-girdle myasthenia (LGM)—A familial disease, mutation to the downstream of kinase 7 (DOK7) gene results in formation of smaller than normal neuromuscular junctions (NMJs). Intravenous administration of rAAV encoding human DOK7 fused to green fluorescent protein (GFP) in Dok-7 transgenic mice caused larger NMJs and longer lifespan ¹⁰⁷.

3.4.3 Duchenne muscular dystrophy (DMD)—Mutation to the dystrophin (DMD) gene causes progressive muscle wasting and death and mainly affects males. A golden retriever model of DMD was treated with rAAV8 encoding U7 small nuclear (sn) RNA that promotes exon skipping to restore a functional in-frame DMD transcript. Treatment was tolerated, with approximately 80% of myofibers expressing truncated, yet functional, dystrophin at the highest dose ¹⁰⁸. A phase I trial with rAAV2.5 (a chimeric AAV2 capsid with 5 mutations from AAV1) injected into the patient's bicep demonstrated no immune response to the vector as well as a safe and tolerated therapy ¹⁰⁹. For **Becker muscular dystrophy (BMD)**, a variant of DMD, the same absence of a T-cell mediated immune response to rAAV1 was observed in a phase I/II trial. Significantly, rAAV1 encoding an

alternatively spliced follistatin demonstrated improvement in 4 out of 6 BMD patients in a six-minute walk test ¹¹⁰.

3.5 Others rare diseases

3.5.1 Smith-Lemli-Opitz syndrome—Mutation to the 7-dehydrocholesterol reductase (DHCR7) gene greatly reduces cellular production of cholesterol and causes systemic issues including learning disabilities, malformed organs, weakened muscles, and many physical abnormalities. Use of rAAV2 or rAAV8 encoding DHCR7 results in disease improvement in mice, with greater efficacy in the rAAV28 treated animals. Significantly, greater therapeutic outcomes were seen in newborn mice versus juvenile animals ¹¹¹, highlighting again that timing may be crucial for treatment of rare diseases that present in childhood.

3.5.2 Lipoprotein lipase deficiency (LPLD)—Following the European Medicines Agency approval of Glybera (rAAV1 encoding human lipoprotein lipase (LPL) for LPLD treatment in 2012, the use of AAV for gene therapy moved into reality. In a recent phase II study of LPLD it was found that treatment with rAAV1 encoding a gain of function LPL variant (S447X) did not elicit immune responses following intramuscular administration. The therapy did not impact safety and found that preexisting antibodies did not effect transgene expression up to 52 weeks after treatment ¹¹².

3.5.3 α -1 antitrypsin (AAT)—Results from a phase II trial (NCT0105433) for AAT disease show transgene expression for more than 1 year without immunosuppression ¹¹³. In this trial, following rAAV1 encoding the α -1 antitrypsin gene (SERPINA1, AAT) treatment, it was found that intramuscular delivery induces regulatory T-cells that attenuate cell-mediated clearance of transduced cells and allows for ongoing transgene expression. Interestingly, immunomodulation may affect initial transduction levels, but may not significantly impact transgene expression afterward ¹¹³. Ongoing phase I and II clinical trials are using rAAV1 encoding AAT with a CB promoter (a cytomegalovirus immediate early enhancer/chicken β -actin promoter with a hybrid chicken β -actin/rabbit β -globin intron), while a phase I trial is using rAAV2 with the same construct. The phase II study reported that all subjects developed anti-AAV antibodies and no subjects developed antibodies against AAT ¹¹⁴. The authors noted that serum levels of AAT > 20 ug/ml were achieved, but that further development will be needed to achieve the required therapeutic levels ¹¹⁴.

3.5.4 Hemophilia B—Impressively, in a phase 1 trial, it was found that intravenous treatment with scAAV8 encoding a codon optimized coagulation factor IX (FIX, F9) with a liver-specific promoter resulted in patient FIX expression for more than 3 years with more than 90% reduction in bleeding episodes ¹¹⁵. Additionally, a long term follow up study is ongoing and will examine patients treated with intrahepatic injection of rAAV2 encoding FIX with an AAT liver-specific promoter in a phase I trial between 2001 and 2004 ²⁹. The study is to be completed in 2019. Importantly, previous trials demonstrated a vector dose-dependent inflammation and loss of transduced hepatocytes by cell-mediated immunity. A phase I/II using rAAV8 encoding FIX aims to overcome the dose-dependent inflammation seen in other trials, where rAAV8 at lower doses allows for FIX expression sooner and

stronger than other serotypes ¹¹⁶. Also promising is preclinical data showing that the use of the scAAV8 encoding FIXR338L, a gain-of-function FIX variant, produced a greater than 6-fold FIX activity compared to wild type FIX without generation of anti-FIX antibodies or CD8+ T cell infiltrates in the liver ¹¹⁷. These data demonstrate a safety and efficacy and support an ongoing scAAV8.FIXR338L phase I/II clinical trial.

3.5.5 Phenylketonuria (PKU)—An autosomal recessive inheritable phenylalanine hydroxylase (PAH) deficiency results in toxic levels of phenylalanine (Phe) in the blood that leads to severe brain damage. Current treatments include diet modification and sapropterin dihydrochloride that helps break down phenylalanine. Significantly, intraperitoneal injection of scAAV8 encoding murine PAH into a PKU mouse model caused reduction in blood Phe to near normal levels. Importantly, this complete phenotypic correction was seen in mice of both genders and lasted more than one year ¹¹⁸.

3.5.6 Glycogen storage disease type Ia (GSDIa)—Buildup of glycogen in the cells of the body impairs function of many tissue types including: liver, kidneys, and small intestines and results in severe hypoglycemia. A naturally occurring canine model of GSDIa was treated with rAAV2/8 and rAAV2/1. Results demonstrated a transient effect with rAAV2/8, with the dogs showing improvement two weeks post treatment, but no longer able to maintain glucose homeostasis two months post treatment. Using the same animal, portal vein injection of rAAV2/1 resulted in maintained glucose homeostasis two months post treatment, lasting up to 23 months. Measurement of lactate levels indicated that a complete phenotype reversal was not achieved, but that a significant improvement encouraged continued investigation for future treatments ¹¹⁹.

3.5.7 Very long-chain acyl-coA dehydrogenase (VLCAD)—Lethargy, muscle weakness, and hypoglycemia result from an autosomal recessive inheritance of VLCAD that prevents fat to energy conversion, specifically mitochondrial fatty acid oxidation. VLCAD-deficient mice were treated via tail vein injection with rAAV9 encoding VLCAD. A significant drop in long-chain fatty acyl accumulation was observed from 2 weeks to 20 weeks post injection. Correction was not achieved in liver tissue, but cardiac tissue showed significant reduction in long-chain metabolites. Following a fasting cold challenge, treated mice maintained body temperature and euglycemia compared to controls that became lethargic and hypoglycemic and had to be euthanized ¹²⁰.

4. EXPERT OPINION

Gene therapy using AAV for treatment of rare disease is rapidly emerging as a major therapeutic option. Advances in vector generation and purification allows for scalable, economic production of clinical quality vectors. Additionally, identification of limiting factors like neutralizing antibodies, vector genome length, and transgene expression are being overcome with multiple solutions. One of the biggest limitations to the study of rAAV gene therapies for rare disease is rooted in a lack of animal models. Pre-clinical testing with *in vitro* models is commonplace, but translation to animals has not kept up with the rate of disease identification. Beyond the traditional Cre/LoxP method to generate disease models in animals, the recent creation of CRISPR-Cas9 mice is a novel method for targeted genome

editing¹²¹. Coupled with next-generation sequencing to identify disease specific genes and the scientific community is empowered with a system to facilitate generation of rare disease models. With promising clinical data suggesting that FDA approval of rAAVs is imminent, the issue of tumorigenicity must be addressed. While numerous trials have demonstrated safety, future research must focus on methods of understanding and preventing nonspecific rAAV genome integration, as the tumorigenicity of the vector in a clinical setting is still not completely understood. However, the recent demonstration that rAAV-mediated promoterless gene targeting without nucleases generates site specific integration in the albumin locus may allow for safe integration in future therapies¹²². Additionally, further research into cellular host responses and rAAV transduction levels may allow for improved therapies. For example, the recently described cyclic GMP-AMP synthase (cGAS) molecule also detects DNA in the cytoplasm and has not been explored in regard to rAAV detection and influence on gene therapy. Additionally, RNA polymerase III (Pol III) is known to link cytosolic DNA detection via DNA-dependent activator of interferon regulatory factor (DAI) to the RNA detection retinoic acid-inducible gene 1 (RIG-I) pathway and promote induction of type I interferon antiviral responses. Inhibition of Pol III has not been explored in a rAAV therapeutic context. It is unclear if other host cell proteins might also be targeted to improve rAAV gene therapy.

The continued research of rAAV for rare disease gene therapy will no doubt bring improved quality of life to the millions of affected individuals if vectors are approved for use. However, in some cases where less than 2,000 persons are affected, it may be difficult to find human patients for clinical trials. Additionally, as with any new therapeutic, it is unclear what long term transgene expression may do to an individual, though some trials have seen expression for more than 6 years with no adverse outcomes. Without a doubt, AAV treatment of rare disease is no longer in its infancy and will soon be a useful tool to improve the lives of countless individuals in the future.

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highlights

- Overview of adeno-associated virus (AAV) biology
- Strategies to generate recombinant AAV vectors to evade immune clearance, improve tissue tropism, and enhance transgene expression
- Historical and current pre-clinical and clinical challenges for AAV-mediated gene therapy
- Ongoing clinical trials with AAV for treatment of rare diseases
- Highlighted pre-clinical and clinical results from AAV studies of retinal, nervous system, lysosomal storage, muscle, and other rare diseases

This box summarizes key points contained in the article

Table 1

Current clinical trials using AAV vectors for treatment of rare disease

Effected tissue / Rare Disease	AAV used	Phase	Status	Sponsor	Trial ID
Retinal					
Choroideremia	rAAV2-REP1	I	Not yet open	Ian M. MacDonald	NCT02077361
Choroideremia	rAAV2-REP1	I/II	Recruiting	University of Oxford	NCT01461213
Leber Congenital Amaurosis	AAV2-hRPE65v2	I	Ongoing	Spark Therapeutics, LLC	NCT00516477
Leber Congenital Amaurosis	rAAV2-hRPE65	I	Recruiting	Hadassah Medical Organization	NCT00821340
Leber Congenital Amaurosis	AAV2-CBSB-hRPE65	I	Ongoing	University of Pennsylvania	NCT00481546
Leber Congenital Amaurosis	rAAV2/4-hRPE65	I/II	Ongoing	Nantes University Hospital	NCT01496040
Leber Congenital Amaurosis	AAV2-hRPE65v2	I/II	Ongoing	Spark Therapeutics, LLC	NCT01208389
Leber Congenital Amaurosis	rAAV2-CB-hRPE65	I/II	Ongoing	Applied Genetic Technologies Corp	NCT00749957
Leber Congenital Amaurosis	rAAV 2/2-hRPE65p-hRPE65	I/II	Ongoing	University College, London	NCT00643747
Leber Congenital Amaurosis	AAV2-hRPE65v2	III	Recruiting	Spark Therapeutics, LLC	NCT00999609
Leber Congenital Amaurosis	tgAAG76 (rAAV 2/2-hRPE65p-hRPE65)	I/II	Ongoing	University College, London	NCT00643747
Leber Congenital Amaurosis	AAV2-hRPE65v2	III	Recruiting	Spark Therapeutics, LLC	NCT00999609
Leber Hereditary Optic Neuropathy	scAAV2-PIND4v2	I	Recruiting	John Guy	NCT02161380
Leber Hereditary Optic Neuropathy	rAAV2/2-ND4	I/II	Recruiting	GenSight Biologics	NCT02064569
Leber Hereditary Optic Neuropathy	rAAV2-ND4	I	Recruiting	Bin Li	NCT01267422
Macular Degeneration	AAV2-sFLT01	I	Ongoing	Genzyme, a Sanofi Company	NCT01024998
MERTK-associated Retinitis Pigmentosa	rAAV2-VMD2-hMERTK	I	Recruiting	Fowzan Alkuraya	NCT01482195
Central Nervous System					
Alzheimer's Disease	CERE-110	II	Ongoing	Ceregene	NCT00876863
Aromatic L-amino Acid Decarboxylase	AAV2-hAADC	I/II	Recruiting	National Taiwan University Hospital	NCT01395641
Parkinson's Disease	AAV-hAADC-2	I	Recruiting	Krystof Bankiewicz	NCT01973543
Parkinson's Disease	AAV2-GDNF	I	Recruiting	National Institute of Neurological Disorders and Stroke	NCT01621581
Parkinson's Disease	CERE-120	I/II	Ongoing	Ceregene	NCT00985517
Late Infantile Neuronal Lipofuscinosis / Batten Disease	AAV2CUhCLN2	I	Ongoing	Weill Medical College of Cornell University	NCT00151216
Late Infantile Neuronal Lipofuscinosis / Batten Disease	AAVrh.10CUhCLN2	I	Recruiting	Weill Medical College of Cornell University	NCT01161576

Effected tissue / Rare Disease	AAV used	Phase	Status	Sponsor	Trial ID
Late Infantile Neuronal Lipofuscinosis / Batten Disease	AAVrh.10CUhCLN2	I/II	Recruiting	Weill Medical College of Cornell University	NCT01414985
Pompe Disease	rAAV9-DES-hGAA	I	Not yet open	University of Florida	NCT02240407
Pompe Disease	rAAV1-CMV-GAA	I/II	Ongoing	University of Florida	NCT00976352
Spinal Muscular Atrophy 1	scAAV9,CB,SMN	I	Recruiting	Jerry R. Mendell	NCT02122952
Blood					
Acute Intermittent Porphyria	rAAV2/5-PBGD	I	Ongoing	Digna Biotech S.L.	NCT02082860
Hemophilia B	AAV2-hFIX16	I	Ongoing	Spark Therapeutics, LLC	NCT00515710
Hemophilia B	scAAV 2/8-LP1-hFIXco	I	Recruiting	St. Jude Children's Research Hospital	NCT00979238
Hemophilia B	AAV8-hFIX19	I/II	Recruiting	Spark Therapeutics, LLC	NCT01620801
Hemophilia B	AskBio009 (AAV8 - Factor IX)	I/II	Recruiting	Asklepios Biopharmaceutical, Inc.	NCT01687608
Liver and Lung					
Alpha-1 Antitrypsin Deficiency	rAAV1-CB-hAAT	I	Ongoing	University of Massachusetts, Worcester	NCT00430768
Alpha-1 Antitrypsin Deficiency	rAAV2-CB-hAAT	I	Ongoing	University of Massachusetts, Worcester	NCT00377416
Alpha-1 Antitrypsin Deficiency	AAVrh.10halphA1AT	I	Not yet open	Weill Medical College of Cornell University	NCT02168686
Alpha-1 Antitrypsin Deficiency	rAAV1-CB-hAAT	II	Ongoing	Applied Genetic Technologies Corp	NCT01054339
Cystic Fibrosis	AAV-CFTR	I	Unknown	National Institute of Diabetes and Digestive and Kidney Diseases	NCT00004533
Systemic					
Familial Lipoprotein Lipase Deficiency	AAV-Human Lipoprotein LipaseS447X	II/III	Ongoing	Amsterdam Molecular Therapeutics	NCT01109498
Familial Lipoprotein Lipase Deficiency	AAV-Human Lipoprotein LipaseS447X	II/III	Ongoing	Amsterdam Molecular Therapeutics	NCT00891306
Metachromatic Leukodystrophy	AAVrh.10cuARSA	I/II	Recruiting	Institut National de la Santé Et de la Recherche Médicale, France	NCT01801709
Muscle					
Becker Muscular Dystrophy	rAAV1.CMV.huFollistatin344	I	Enrolling by Invitation	Nationwide Children's Hospital	NCT01519349
Limb girdle muscular dystrophy type 2C	scAAVrh74.MCK.hSGCA	I/II	Ongoing	Jerry R. Mendell	NCT01976091