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Adeno-associated virus structural biology as a tool in vector development

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Abstract

Adeno-associated viruses (AAVs) have become important therapeutic gene delivery vectors in recent years. However, there are challenges, including intractable tissues/cell types and pre-existing immune responses, which need to be overcome for full realization of this system. This review addresses strategies aimed at improving AAV efficacy in the clinic through the creation of hybrid vectors that display altered or more targeted specific tissue tropisms, while also escaping recognition from host-derived neutralizing antibodies. Characterization of these viruses with respect to serotypes contributing to their capsid, using available 3D structures, enables the identification of regions critical for particular tropism and antigenic phenotypes. Structural information also allows for rational design of vectors with specific targeted tropisms for improved therapeutic efficacy.

Keywords

adeno-associated virus; capsid structure; chimera; directed evolution; gene therapy; rational design; tissue tropism

Gene therapy

The strategy of gene therapy involves either replacing a defective gene with a functional one, or the downregulation of a dysfunctional gene in order to block its deleterious effects. Many of the clinical gene therapy trials conducted to date have sought treatments for monogenic diseases, which are caused by mutations in a single gene product [1]. However, many conditions such as diabetes, heart disease and neurological disorders are multifactorial in nature, and will prove a challenge to treat using gene replacement alone. Some of the nonviral gene-delivery methods currently in use involve the electroporation of naked DNA or the use of lipoplexes, in which DNA is protected inside a liposome shell [2,3]. These nonviral vectors have the advantage of being able to deliver larger genes and producing low host immunogenicity, but suffer from low transgene expression that is short lived. The most promising method of gene delivery uses viruses, which have evolved to be highly efficient at infecting specific host cell types and delivering and expressing their genomic payload. The most widely used viral vectors are derived from adenoviruses, retroviruses and parvoviruses,

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and each have been shown to be highly effective in both gene delivery and expression. In previous studies involving adenoviral vectors, they have been shown to produce robust unwanted immune responses in human subjects, while some lentiviral vectors have been associated with insertional mutagenesis and the development of cancer [4,5]. However, adeno-associated viruses (AAVs), belonging to the parvovirus family, have demonstrated a favorable safety profile [1]. Taken together with their ability to package and express transgenes in a variety of tissues types, this family of viruses has emerged as a powerful tool for therapeutic gene delivery [1,6,7]. For the purpose of this review, we will focus on the development of AAV vectors for their use in gene therapy aided by structural information.

AAV background

AAV is a small, nonenveloped icosahedral virus approximately 260 Å in diameter [8]. The AAVs belong to the *Dependovirus* genus of the *Parvoviridae* family and are considered to be replication deficient due to a requirement for a helper virus, such as adenovirus or herpesvirus, for genome expression and replication. It contains a 4.7-kb ssDNA genome, consisting of three open-reading frames (ORFs) flanked by 145 base pair inverted terminal repeats (ITRs) (**Figure 1**). The *rep* ORF encodes the *rep* gene, which is responsible for the expression of four non-structural proteins (Rep78, Rep68, Rep52 and Rep40). These Rep proteins are made from alternative splicing of transcripts from the P5 and P19 start sites (**Figure 1**), and although they are required for viral replication, they are not sufficient to generate a productive infection. Rep78 and Rep68 have been shown to possess site-specific endonuclease activity and are necessary for viral DNA replication and site-specific integration into the host genome. Although all four Reps contain helicase and ATPase activity, the smaller Reps are indispensable for genome packaging. The *cap* ORF contains the single *cap* gene and produces three overlapping structural proteins (VP1, VP2 and VP3) from the P40 promoter by alternative splicing and the usage of an alternative start codon (**Figure 1**). Sixty copies of these three VP proteins interact in a 1:1:10 ratio to form the T = 1 viral capsid. A newly identified AAP, translated from an alternative ORF in the VP2/VP3 mRNA, assists in capsid assembly [9–11].

The AAV life cycle consists of many stages, each of which presents a possible barrier to efficient infection [12]. The first step of infection involves AAV binding to the target cell via the primary attachment receptor and serotype AAV2 accomplishes this using heparan sulfate proteoglycan (HSPG) [13]. For AAV2, the HSPG-bound virus also requires one or more of five known coreceptors including $\alpha 5\beta 1$ integrin, $\alpha V\beta 5$ integrin, HGF receptor, laminin receptor or FGF receptor type 1 to enter the host cell [13–18]. There are many different receptors and coreceptors involved in the attachment process for each of the AAV serotypes, thus accounting for the broad range of tissue tropisms. Next, AAV undergoes receptor-mediated endocytosis and internalization occurs via clathrin-coated pits in a dynamin-dependent process [19], although a clathrin-independent mechanism has also been described [20]. Once inside the host cell, the AAV capsid must undergo vesicular trafficking through the endosomal pathway. This step is crucial to the transduction process because the viral capsid appears to be modified by the drop in pH in the endosome, which primes the virus for nuclear transport and uncoating. Structural changes in the AAV capsid trigger the externalization of a conserved phospholipase A₂ (PLA₂) motif present on the unique N-terminal domain of the VP1 protein (VP1u) [21–23]. This step is important for successful infection and it is believed to aid in viral escape from the endosome. Concurrently, the exposure of nuclear localization signals located in the VP1u and VP1/VP2 N-termini are crucial for trafficking of the AAV capsid to the nucleus [24,25]. Recent studies have shown that AAV virions can interact with molecular motors on microtubule networks to facilitate perinuclear accumulation of capsids [26]. However, the method by which the virus enters the nucleus is uncertain. Once inside the nucleus, the virus uncoats, releasing its genomic

ssDNA and the infection proceeds in either a lytic or lysogenic manner [22,27]. In the presence of a helper virus, the lytic infection results in genome replication, viral gene expression and the production of Rep, Cap and AAP proteins. Cap proteins assemble into viral particles with the help of AAP and Rep packages the AAV genome into the preformed capsids [6,28]. However, in the absence of a helper virus, AAV can persist in an episomal form as DNA concatamers, or may integrate site specifically into chromosome 19q13.4 at low levels [29,30].

Currently, 13 distinct human and nonhuman primate AAV serotypes (AAV1–AAV13) have been sequenced and PCR studies of both nonhuman primate and human tissues have identified numerous other AAV genomes [31–42]. The AAVs are classified into six genetic groups (clades A–F) and two clonal isolates (AAV4 and AAV5) based on antigenic reactivity and sequence comparison (**Figure 2**) [41]. Comparing the AAV serotypes to one another, they share approximately 65–99% sequence identity and 95–99% structural identity (percentage of superposable C α positions). These AAVs differ dramatically in their tropism for various target tissues, including cardiac and skeletal muscle, liver and lung tissue, and cells in the CNS [41,43]. These differences can be exploited for use in gene therapy, enabling the directed treatment of specific tissues.

AAV structure

The AAV serotypes demonstrate unique tissue tropisms, as well as other characteristics including differential transduction efficiencies and blood-clearance properties [1,44–46]. These unique properties are likely dictated by their viral capsid structure. Using x-ray crystallography or cryo-electron microscopy and image reconstruction (cryo-reconstruction), the structures of AAV1–AAV9 have been determined [47–56] [GOVINDASAMY L, MILLER E, AGBANDJE-MCKENNA M, UNPUBLISHED DATA]. In all of these structures, only the VP3 common region is observed, which is likely due to disorder in the VP1/VP2 common region [57] and/or the low copy number of the VP1 unique region and VP1/2 common regions, which is incompatible with icosahedral symmetry utilized during structure determination [8].

The AAV VP3 structure contains highly conserved regions that are common to all serotypes, a core eight-stranded β -barrel motif (β B– β I) and a small α -helix (α A) (**Figure 3A**) [47–56]. The loop regions inserted between the β -strands consist of the distinctive HI loop between β -strands H and I, the DE loop between β -strands D and E, and nine variable regions (VRs), which form the top of the loops. These VRs are found on the capsid surface (**Figure 3B**) and can be associated with specific functional roles in the AAV life cycle including receptor binding, transduction and antigenic specificity [48,58–67]. Thus, these structural differences between the serotypes can be exploited for designing tropisms to specific tissues or cell types for the treatment of specific diseases.

In an assembled capsid, the VP monomers interact at icosahedral (two-, three- and five-fold) symmetry axes (**Figure 4A**) [47–56]. A monomer (**Figure 4B**) interacts with another to form the twofold axis of symmetry (**Figure 4C**) that is marked by a depression, and this region is believed to be responsible for conformational changes in the capsid that arise during viral endosomal trafficking [52]. The threefold axis of symmetry (**Figure 4D**) occurs where three monomers interdigitate and form spiky protrusions. This region has been shown to be important for receptor binding and antibody recognition [59–67]. Five VP monomers interact to form a pentamer (**Figure 4E**) with a characteristic central pore and surrounding canyon region. This pore is the only channel that spans the capsid, linking the inside of the virus to the outside environment. This site is postulated to serve as the portal for VP1/VP2 externalization (for necessary PLA₂ and nuclear localization signal functions) and also for genome packaging [21–25,68].

In recent years, structural studies have provided increasing insight into the AAV life cycle. For example, cryo-EM and image reconstruction of AAV2 complexed with its heparan sulfate (HS) receptor [62,64] provided a better understanding of the HSPG receptor binding site previously identified by mutagenesis experiments [61,63,65]. The complexed structures showed that the heparan footprint consists of residues R484, R487, K532, R585 and R588 (AAV2, VP1 numbering), which are located on the inside surface of the threefold protrusions [62,64]. Other studies have sought to mimic the conditions that the virus encounters while trafficking through the cell in order to gain insight into capsid structural transitions [52]. Briefly, AAV8 capsids were incubated at pH 4.0, pH 5.5, pH 6.0 or pH 7.5 (after previous incubation at pH 4.0) and then their structures were determined by x-ray crystallography to identify regions of the capsid that underwent conformational changes. Two regions of interest were described: one on the interior surface of the threefold icosahedral axis and the other on the exterior of the capsid at the twofold symmetry axes, dubbed the 'pH quartet'. At low pH, it was found that the capsid underwent several conformational changes, effectively disrupting both its interaction with nucleic acid density and also weakening the twofold region. This suggests that the low pH associated with endosomal trafficking triggers destabilization of the capsid, which may allow for the externalization of the VP1 PLA₂ domain and also primes the capsid for uncoating.

AAV as a gene delivery vector

The AAVs are attractive gene delivery vectors because they have the ability to package and express foreign genes in a broad range of tissues [6,7,45]. Different AAV serotypes display specific tissue tropisms and varying transduction efficiencies, and have been shown to achieve stable, long-term gene expression. They are able to infect both dividing and nondividing cells without any known associated pathogenicity. Additionally, recombinant AAVs (rAAVs) can be manufactured and purified at a high titer, making them readily available for clinical use.

In the manufacture of rAAV gene therapy vectors, two approaches are utilized. For the first, a triple transfection is employed, whereby three separate plasmids are used to transfect mammalian cells in culture [6]. One plasmid contains the therapeutic gene of interest flanked by the viral ITRs, which is the only *cis* signal necessary for packaging into the AAV capsid [69,70]. A separate plasmid encodes the *rep* and *cap* genes, while the final plasmid contains adenoviral helper genes that are necessary for viral replication [71]. The second approach uses a baculovirus/Sf9 system in which three separate viruses (Rep baculovirus, VP baculovirus and an AAV ITR flanking a gene of interest baculovirus) are used for infection [72–74]. The resulting rAAV vectors contain the transgene packaged into the rAAV capsid of choice.

One of the drawbacks of using AAV for therapeutic gene delivery is its relatively small packaging capacity [6]. Studies in recent years have shown that the largest gene product that can be efficiently packaged into the AAV capsid is approximately 5 kb in length [75–78]. However, other approaches have tried to circumvent this packaging issue by splitting the transgene cassette between two rAAV vectors containing splice donor and splice acceptor sites [79]. Once the host cell is infected, recombination of the ITRs results in splicing of the mRNA transcript and expression of a functional protein product. This approach has shown that it is possible to deliver therapeutic genes up to 9 kb in size, although at a lower efficiency than their nonspliced counterparts.

AAV in clinical trials

There are currently approximately 1900 gene therapy clinical trials that have been initiated worldwide, 99 utilizing AAV as their vector of choice [80]. Many of the trials have used rAAV2 as the delivery vector [1,45,80]. These trials have resulted in the successful treatment of several inherited diseases, including a rare form of blindness called Leber's congenital amaurosis (LCA) [81–83]. LCA is a degenerative disorder of the retina caused by a mutation in one of 14 retinal genes and ultimately results in early-onset blindness. One rAAV2 LCA therapy trial delivered *RPE65*, a retinal pigment epithelium gene, by subretinal injection. This therapy was well tolerated by patients and resulted in an improvement of visual function. Another clinical trial used rAAV8, a liver tropic serotype, to deliver Factor IX for the treatment of hemophilia B [84,85]. This is a severe blood-clotting disorder caused by mutations in the gene for blood coagulation Factor IX, which manifests in spontaneous hemorrhaging. The Factor IX transgene, delivered to the liver, resulted in correction of the bleeding phenotype with few observed side effects.

In late 2012, the European Commission approved a rAAV vector as the first gene therapy treatment for clinical use in the Western world [86,201]. This vector Glybera® (alipogene tiparvovec, UniQure), utilizing rAAV1, a muscle tropic serotype, was developed to treat a rare, inherited disorder called lipoprotein lipase (LPL) deficiency. Lipoprotein lipase deficiency is caused by a defective *LPL* gene, which results in an inability to break down fats, leading to high levels of fat in the blood and may ultimately result in severe pancreatitis. Glybera works by introducing a normal copy of the *LPL* gene into the patient. Clinical trials of this therapy demonstrated that it was well tolerated by patients and it was capable of producing long-term expression of the therapeutic LPL enzyme.

Challenges to AAV gene delivery

In spite of its success, significant challenges to AAV vector gene-delivery applications have been highlighted in studies using animal models and in several clinical trials. These include the detrimental effects of the pre-existing host immunity to AAV capsids and new responses to the expressed transgene [1,87], and difficulties in delivering genes efficiently to certain target cells types, such as hematopoietic stem cells, tumor cells and pancreatic islet cells [88–92]. Pre-existing neutralizing antibodies (NABs), present in a high proportion of the population who have been previously exposed to the virus, can hinder AAV gene delivery. It has been estimated that approximately 70% of the human population are seropositive for AAV2 NABs and to a lesser extent for NABs to the other AAV serotypes [87,93,94]. Studies with large animals as well as rodents show that the antibody response to the AAV capsid efficiently blocks gene transfer, even at low levels [94,95]. Consequently, there has been an increasing interest in the development of AAV variants isolated from animals, mostly nonhuman primates, for human gene delivery, since ideally most humans would not have a pre-existing NAB response against them. One such example is AAVrh32.33, an AAV variant derived from rhesus macaque isolates [93]. This isolate exhibits a remarkably low seroprevalence among humans, and for this reason may prove to be a useful gene therapy vector. In addition to the humoral immune response, it has been shown that AAV gene therapy is capable of provoking a T-cell response to the capsid, leading to a loss of therapeutic efficacy and preventing readministration with the same vector [1,45,84].

Efforts towards understanding the antigenic structure of the AAV capsid have included peptide mapping of antigenic regions [66,94], as well as structure determination of AAV capsids complexed with fragment antibodies (Fabs) generated from mouse monoclonal antibodies that neutralize infection [58–60]. Studies by Moskalenko *et al.* demonstrated that the AAV2 capsid contains several immunogenic regions [94]. These epitopes were shown to

cluster around the twofold depression, the threefold protrusions, and at the fivefold pore and the surrounding canyon area. Wobus *et al.* reported the peptide epitopes for a number of antibodies to the AAV2 capsid, which also map to similar regions of the capsid [66]. Only recently were the structures of several AAV capsids complexed with corresponding capsid antibodies reported [58–60]. These structures were determined by cryo-electron microscopy and image reconstruction, and interpreted by molecular docking of the available capsid structures and homologous models for the bound Fab fragments. An analysis of the AAV2:A20 complex structure mapped the antibody epitope to the capsid surface between the two- and five-fold axes [58], showing overlap with residues identified by site-directed mutagenesis [63]. The AAV8:ADK8 structure, combined with mutagenesis, identified an epitope on the protrusions that surround the threefold axis of symmetry. Further studies showed that knocking out the ADK8 epitope created a capsid variant capable of escaping neutralization while maintaining tissue tropism [96]. A recent structural analysis of AAV antibody-binding sites examined several monoclonal Fabs directed against the capsids of AAV1, AAV2, AAV5 and AAV6 [59]. Specifically, 4E4 and 5H7 were found to interact with AAV1 and AAV6 at the protrusions surrounding the threefold axis of symmetry. An equivalent binding profile was observed with AAV2:C37-B, similar to what was seen with AAV8:ADK8. In contrast, 3C5 binding to AAV5 occurred in the canyon area bordering the two- and five-fold axes, much like AAV2:A20. These results demonstrated that antibodies interact with the capsid at common binding footprints (at the threefold protrusions or in the fivefold canyon) and that these are coincident with some receptor binding sites and determinants of transduction efficiency. These studies point to potential mechanisms for virus neutralization by antibodies, and thus underlie the usefulness of structural information for AAV capsid engineering for improved therapeutic utility.

Another major challenge to AAV gene therapy is the ability to direct viral tissue tropism. Although AAV serotypes have been shown to exhibit differential tissue tropisms, they also have the ability to cause off-target effects and often accumulate in the liver [43]. In addition, clinical trials for hemophilia B found that the AAV2 vector stimulated a cytotoxic T lymphocyte response, leading to liver toxicity in human patients [84,85]. For this reason, there is a need to develop next-generation gene therapy vectors that will enable the transduction of specific tissues, which are resistant to transduction by naturally occurring AAV serotypes, or to limit the virus tropism to certain tissues. Several methods such as directed evolution and rational capsid engineering have been developed in recent years to address these challenges and also to create vectors that will evade the pre-existing human immune response [45].

Chimeric AAV variants

Chimeric viruses can be defined as “containing capsid proteins that have been modified by domain or amino acid swapping between different serotypes” [97–100]. Chimeric rAAVs are created either by directed evolution or by rational capsid engineering of variable surface amino acids on the AAV capsid. The directed evolution approach involves using either error-prone PCR or DNA shuffling to evolve the capsid proteins. Error-prone PCR utilizes specific conditions to enhance the error rate of the polymerase to generate capsid variants with random mutations. On the other hand, DNA shuffling uses restriction enzymes to digest the *cap* genes of selected sero-types. These fragments are then allowed to recombine, at random, into full-length capsid genes and then amplification of the variants occurs. Either method can be used to create a chimera, followed by a selective screening procedure with the preferred cell type in order to isolate mutants expressing the desired characteristics. Finally, the capsid engineering approach uses structural and sequence information to design new chimeric viruses by transferring specific capsid residues or domains (such as VRs) from one serotype to another to confer a desired phenotype.

In the following sections, some examples will be given of AAV chimeras either created by capsid evolution or by rational capsid engineering. Homology models were generated for each chimera based on mutant sequence data and available Protein Data Bank coordinates for the VP3 structures of AAV1, AAV2 and AAV6–9 (Protein Data Bank ID – AAV1: 3NG9; AAV2: 1LP3; AAV6: 3OAH; AAV7: unpublished; AAV8: 2QA0; AAV9: 3UX1). The contributions from the relevant coordinate files were combined, renumbered (when required), the geometry regularized and visualized in the program Coot [101]. The coordinates for a full capsid were generated from the individual coordinate files by icosahedral matrix multiplication using the Oligomer generator subroutine in VIPERdb [102]. The Chimera software package [103] was used to color the capsid amino acid residues according to their contributing serotype (e.g., AAV1: purple; AAV2: blue; AAV6: hot pink; AAV7: cyan; AAV8: green; AAV9: brown). Additionally, ‘Roadmap’ images were generated using Radial Interpretation of Viral Electron density Maps (RIVEM) software [104], and altered residues were color coded by their contributing AAV serotype. The Roadmap figures illustrate the 2D projection of the amino acid residues present on the capsid surface within the context of a viral asymmetric unit. By analyzing the structures of these hybrid AAVs, we can develop a better understanding of the contributions of each serotype to the observed viral phenotype (such as tissue tropism and antigenicity).

Chimeras made by directed evolution

AAV-DJ—This chimera was created to have enhanced tropism for liver and resistance to pre-existing Nabs, and was made using a two-pronged approach [105]. First, the capsid genes of eight different AAVs (AAV2, AAV4, AAV5, AAV8 and AAV9, and animal isolates AAVV, BAAV and CAAV from avian, bovine and caprine species, respectively) were combined to create a shuffled AAV capsid library. The library was serially screened on human hepatoma cells (Huh-7 and HepG2), which naturally have a low permissiveness for AAV infection. The resulting clones underwent further selection in the presence of pooled human antisera (intravenous immunoglobulin), which have a neutralizing effect against many AAV serotypes, and then were amplified on the hepatoma cells for several more rounds of directed evolution. The AAV-DJ chimera (**Figure 5A**), containing capsid VP contributions from AAV2, AAV8 and AAV9, has enhanced liver tropism *in vivo* in both naive mice and mice treated with intravenous immunoglobulin, an ability to evade known NABs and can efficiently deliver the Factor IX transgene to liver tissue. The structure of AAV-DJ, by cryo-reconstruction, showed conservation of the HS receptor attachment site of the parental AAV2 combined with structural difference in VR-I (contributed by AAV9), a loop known to be involved in binding the A20 NAb [106]. Thus, cellular recognition is maintained and the variation provides an explanation for the chimera’s ability to evade recognition from A20 and potentially other NABs that bind at VR-I [59]. The story of AAVDJ demonstrates that it is possible to combine the best parts of known AAV serotypes, such as AAV2’s high level of expression in liver tissue and HSPG receptor binding with AAV8/AAV9’s enhanced liver transduction, and the ability to evade pre-existing NABs to generate a novel AAV chimera using directed evolution.

AAV-HAE1 & AAV-HAE2—These variants were isolated after an attempt to identify AAV chimeras with a tropism for ciliated airway epithelial cells for the treatment of patients suffering from cystic fibrosis [107]. The approach used a combinational AAV library, generated from shuffling of AAV1–9 (excluding AAV7) capsid genes, followed by directed evolution in primary human airway epithelial (HAE) cells. The chimeras contain capsid components from AAV1 and AAV6 (AAV-HAE1), or AAV1, AAV6 and AAV9 (AAV-HAE2). The available structures of the AAV serotypes were used to delineate the VP regions contributed from each virus and thus the functional regions that were conferred to the chimera. AAV-HAE1 (**Figure 5B**) and AAV-HAE2 (**Figure 5C**) varied the most at the

six amino acid positions that differ between AAV1 and AAV6, the two serotypes contributing the most sequence to the chimeras. Five of the six residues are within the VP3 common region and localized at the icosahedral threefold axis of the capsid, with three residues on the exterior surface and two on the interior surface (**Figure 5B & C**). The AAV-HAE1 chimera contained one exterior residue difference from AAV1 and two from AAV6, and both residues on the inside of the capsid were from AAV1. On the other hand, AAV6 contributed all three of the exterior surface residues of AAV-HAE2, and AAV1 and AAV6 each contributed to the changes in the interior. The AAV1/6 difference at amino acid position 129, for which there is no structural information, was contributed by AAV9 for HAE-2. Further characterization of these chimeras on patient HAE cells showed that they were able to transduce roughly three times better than the parental AAV6 and were also able to correct the cystic fibrosis defect. These results suggested that these five residues could combine in different ways to confer improved tropism for HAE cells. The use of the structures of AAV1 and AAV6 for comparison of these chimeras thus provided understanding of the contribution of specific amino acid residues to their improved transduction phenotype.

AAVM41—This chimera was created by directed evolution to obtain a vector with increased tropism for muscle [100]. It was generated using a combinatorial AAV library created by DNA shuffling (of AAV1–AAV9 capsid genes except for AAV5) followed by direct *in vivo* screening in mice to isolate clones that were enriched in the muscle, but scarce in the liver. The M41 myocardium-tropic variant is derived from AAV1, AAV6, AAV7 and AAV8 capsid components (**Figure 5D**). The structural contribution of these serotypes was analyzed using a 3D homology model generated by combining the crystal structure or model coordinates for the respective VP3 amino acid contributions from AAV1, AAV7 and AAV8 or AAV6. The twofold region of the capsid surface was contributed by AAV6, the threefold by AAV1 and AAV6, and the fivefold channel and canyon floor by AAV7 and the HI loop by AAV6. Contributions by AAV8 were localized to the interior of the capsid. *In vitro* testing in mice demonstrated that AAVM41 was more efficient than the parental AAV6 in transducing heart muscle, suggesting that surface contributions from the other serotypes help to confer this phenotype. Following systemic delivery of AAVM41 expressing delta-sarcoglycan in a cardiomyopathy hamster model, this chimera achieved long-term transgene expression and rescue of cardiac functions. This study suggests that AAVM41 has a unique phenotype due to its contributing parental serotypes and that juxtaposition of capsid residues conferred improved receptor binding to muscle and decreased binding to liver.

AAV-1829—This chimera was generated in an effort to overcome previous observations that melanoma cells have low permissiveness to AAV infection [18,91]. A chimeric capsid library, generated by shuffling of the capsid genes of AAV1–9 [91] was used to select the chimera in CS-1 melanoma cells. The chimera was derived from parental AAV1, AAV2, AAV8 and AAV9, hence its name. A 3D structural model of the AAV1829 chimera (**Figure 5E**) showed that the fivefold region was primarily composed of AAV1 residues, while AAV2 residues compose the characteristic HI loop. In addition, AAV2 contributes the surface-exposed variable loops at the threefold axis of symmetry, including residues previously shown to be involved in HS binding [61,65]. AAV8 residues were also shown to contribute to minor protrusions surrounding the threefold axis. Most of the twofold region is composed of residues from AAV2 and AAV9. Compared with AAV-1829, none of the parental serotypes were able to infect the CS-1 cells with as high of an efficiency, demonstrating that this chimera was unique. Combining information from structural analysis and rational mutagenesis studies, it was determined that the C-terminal AAV9 residues are likely to be important in dictating tropism for melanoma cells. In addition, AAV-1829 was found to utilize HS as a primary receptor, like AAV2, and demonstrated a unique

immunological profile compared with its parental serotypes. In a mouse model, this chimera has a unique tropism for melanoma cells and a reduced affinity for muscle, liver and neuronal cells compared with its parental serotypes. Thus, this highly targeted vector may prove to be very effective in the treatment of melanoma by AAV-mediated gene therapy.

Chimeras made by rational capsid engineering

AAV2 Y/F & AAV2 T/V mutants—In recent years, trafficking experiments have shown that AAV2 capsids are often targeted to the proteasome, leading to capsid degradation and a decrease in viral transduction efficiency [108]. Tyrosine, serine and threonine residues on the capsid surface are capable of undergoing phosphorylation and subsequent ubiquitination, targeting the virions to the proteasome. Careful examination of the AAV2 capsid structure found seven surface-exposed tyrosine residues, Y252, Y272, Y444, Y500, Y700, Y704 and Y730 (AAV2 VP1 numbering), which were mutated to phenylalanine in order to determine their roles in phosphorylation (**Figure 6A**) [108]. Alteration of these residues resulted in a significant increase (30-fold) in transduction over wild-type AAV2 in murine hepatocytes *in vivo* at a log lower dose and ultimately led to the development of next-generation AAV vectors with higher transduction efficiencies at lowered administered doses [109]. In a follow up experiment, Aslanidi *et al.* sought to determine the contribution of each of the 17 surface-exposed threonine residues to AAV2 transduction efficiency [110]. Mutagenesis of the capsid surface threonine residues to valines and subsequent *in vitro* studies demonstrated that T455V, T491V, T550V and T659V were responsible for an increase in the transduction efficiency of AAV2. Simultaneous mutation of several previously identified tyrosine residues and one threonine residue produced a variant (Y444F + Y500F + Y730F + T491V) capable of transducing target cells approximately 24-fold more efficiently than wild-type AAV2 (**Figure 6B**).

AAV2 ↔ AAV8 loop variants—In order to gauge the importance of AAV surface capsid residues on transduction efficiency, residues located in several VRs of the capsid that compose the threefold protrusions were targeted for modification [96]. Single residues in these regions were swapped from AAV2 to the AAV8 *cap* gene or *vice versa*. Residues 581–584 and 589–592, located in the GH loop (and VRIII), were found to be significant transduction determinants of both the liver and heart (**Figure 6C**). This study also discovered that peptide insertions at position 590 in the AAV8 capsid were tolerated and that they could lead to efficient retargeting of the virus to certain tissues.

AAV2i8—This chimera was one of the first examples to be created by the capsid-engineering approach guided by structure and based on functional information [44]. It was engineered to improve AAV tropism for the skeletal and cardiac muscle, while also detargeting the virus from the liver. Briefly, six amino acids that were previously shown to be involved in AAV2-HS binding were substituted with the corresponding domain from AAV8 [61,62,65]. This engineering removed two critical basic residues – R585 and R588 – from the AAV2 capsid surface (**Figure 6D**). *In vivo* studies in mice showed that this chimera was able to transduce skeletal muscle much more efficiently and at lower doses than the wild-type AAV8. In addition, AAV2i8 displayed an altered antigenic profile compared with the parental viruses, and was able to traverse blood vasculature more efficiently than the parental serotypes. All of the aforementioned properties of AAV2i8 add to the growing body of data indicating that AAV chimeras acquire synergistic properties from their parental serotypes. AAV2i8 is a promising candidate for the future treatment of musculoskeletal disorders.

AAV9 variants—In addition to the creation of chimeras from several AAV serotypes, efforts to engineer AAV variants have also focused on the alteration of amino acids within a

particular serotype. Studies by Pulicherla *et al.* attempted to modify the broad tissue tropism of AAV9 by using a combination of several approaches guided by structure information [98]. Error-prone PCR was used to create a diverse AAV9 capsid library with mutations specifically generated in the GH loop region (amino acids 390–627) of the VPs. This sequence was chosen for modification because the GH loops from three monomers interdigitate to form the protrusions at the threefold axis of symmetry, and previous studies have shown this region to be critical for receptor binding, influencing tissue tropism and antigenicity. Mapping of the variants obtained onto an AAV9 structure model showed that most of the mutations clustered on the outer surface of the capsid. Further characterization of selected mutants in mice, using a systemic tail vein injection, identified, among other phenotypes, two variants (9.45 and 9.61) that transduce cardiac and skeletal muscle as efficiently as AAV9, but displayed a 10–25-fold decrease in liver transduction. These variants contained changes at amino acid positions N498Y and L602F for AAV9.45 and N498I for AAV9.61, all located at the icosahedral three-fold region (**Figure 6e**, changes shown in black). This study identified a critical capsid determinant of AAV9 liver transduction and demonstrated that it is possible to generate AAV vectors with markedly reduced tropism for certain tissue types, while maintaining efficient transduction of target tissues.

AAV2.5—This is the first example of an AAV chimera generated using a structurally guided rational design strategy. The vector was designed to have a muscle-tropic phenotype while evading the immune response of the parental vectors for the treatment of Duchenne muscular dystrophy [97]. The vector engineered the muscle-tropic properties of AAV1 onto the AAV2 capsid, which displays a favorable safety profile and is easy to purify. The first step in the rational design process involved the identification of AAV serotypes that display muscle tropism, for example, AAV1, AAV7, AAV8 and AAV9. These serotypes were then compared with AAV2 and AAV3b, with low muscle transduction efficiency, using an amino acid sequence alignment as well as structural comparison. Sequence and structural regions that are conserved or differed between the viruses were then identified. AAV1 amino acids were then substituted into AAV2 at positions that differ between AAV2 and AAV3b and the muscle-tropic serotypes.

AAV2.5 consists of an AAV2 backbone with four point mutations (Q263A, N705A, V708A and T716N) and one single residue insertion (T265) from AAV1 (**Figure 6F**). Two of the changes (Q263A and T265, shown in dark purple) occur in VR-I, while the remaining three (N705A, V708A and T716N, shown in light purple) are located in the VR-IX loop. The AAV2.5 vector maintains the HS-binding properties of parental AAV2 and efficiently transduces HeLa, Cos and 293 cells much like its parent AAV2, but gains the skeletal muscle transduction properties of AAV1. In addition, the two VRs, VR-I and VR-IX, form part of the A20 NAb epitope and confer an A20 escape phenotype onto AAV2.5. Compared with parental AAV1 and AAV2, this vector demonstrates a unique immunological profile. These properties allow AAV2.5 to transduce muscle tissue efficiently in patients even in the presence of pre-existing NAb.

AAV2.5 has been evaluated in a Phase I clinical trial for the treatment of patients suffering from Duchenne muscular dystrophy. This severe form of muscular dystrophy is inherited and caused by mutations in dystrophin, a cytoskeletal protein of the muscle. The disease course involves progressive muscle degeneration, eventually leading to premature death. In this study, the AAV2.5 hybrid vector was used to deliver a miniaturized dystrophin gene to six human subjects. The vector achieved robust gene transfer in skeletal muscle and displayed a safe profile in the patients. The AAV2.5 vector paves the way for future generations of structurally guided designer AAVs for use in therapeutic gene delivery.

Conclusion

Naturally isolated AAV variants as well as chimeric capsids, modified to capture desirable serotype features such as enhanced or specific tissue tropism and host immune response escape, are highly promising as vectors. The engineered vectors are particularly interesting because they can target specific tissues/cells for transgene delivery at lower administered dosages compared with wild-type serotypes [45,99]. They may also demonstrate a different immunological profile than either parent virus, thus encountering little to no interference from pre-existing NABs.

Comparing the properties of some of the natural AAV isolates and chimeras allows a better understanding of the roles the VRs play in dictating tissue tropism, antigenic sites and other phenotypic properties (**Table 1**). Correlation of the VP1 amino acid sequences and the available structural information for the VP3 common polypeptide from different serotypes, along with known infection phenotypes, enables the dissection of the functional regions of the AAV capsid. These regions include those that are critical for receptor attachment and cellular entry, endocytic trafficking transitions, transduction efficiency, capsid uncoating, capsid assembly, genome packaging and recognition of host antibodies. This information enables the engineering of vectors with enhanced transduction for specific tissue targeting and detargeting, and thus individualized disease treatment. It also allows for the creation of host immune system response escape variants. These features are required to generate an AAV vector toolkit with therapeutic efficacy and are essential for full realization of this promising AAV gene delivery system in the clinic. From the current data, we can conclude that the AAV capsid is multifunctional in nature and still holds many secrets that have yet to be uncovered. More studies will need to be conducted in order to identify specific amino acids that are responsible for a desired AAV vector phenotype, with the eventual goal of creating an AAV vector toolkit that can be customized for use in the clinic.

Future perspective

As new AAV chimeras are created over the coming years and their phenotypes are analyzed, a better picture will begin to emerge of the roles of the VRs on the capsid surface. Structural annotation of the AAV variants will allow us to home in on the importance of certain amino acid residues. Increasing information will become available about specific cellular receptors and antigenic reactivity to the AAV capsid and the specific amino acids that mediate their interactions. Lessons learned from animal and human gene therapy studies can teach us the importance of limiting tissue tropism, the need for tissue detargeting, vector delivery methods and the intricacies of the immune system. Taken together, this vast amount of information needs to be deposited into an AAV gene therapy data bank and thus available to be utilized for the rational capsid design of future AAV vectors. With this knowledge, it could be possible to target tissues that are currently refractory to AAV transduction at very low administered vector doses and also circumvent the host immune response at the same time. The availability of whole-genome sequencing will ensure that blood and tissue samples taken from patients can be screened in order to make vectors tailored to individual treatment needs. The dream of using AAV vectors to deliver therapeutic treatments for a variety of diseases will become a reality.

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■ of interest

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Executive summary

Adeno-associated virus background

- Adeno-associated viruses (AAVs) are nonenveloped T = 1 icosahedral viruses approximately 260 Å in diameter.
- The AAV capsid encloses a 4.7-kb ssDNA genome, consisting of three open-reading frames: *rep*, *cap* and *aap*.
- The *cap* gene expresses three structural proteins (VP1, VP2 and VP3) that interact to form the capsid.
- AAV capsid attachment to cellular receptors plays a role in tissue tropism.
- Currently, 13 AAV serotypes have been identified and these demonstrate tropism for various target tissues. The observed tropism differences among AAV serotypes can be exploited for use in gene therapy.

AAV structure

- The AAV VP3 monomer structure contains a highly conserved core region consisting of eight antiparallel β -strands and an α -helix, with loop insertions between the β -strands forming nine distinct variable regions (VRs).
- The VRs are found on the capsid surface and can be associated with specific functional roles in the AAV life cycle, such as receptor binding, transduction and antigenic specificity.
- Sixty copies of the VP monomers interact at icosahedral (two-, three- and five-fold) symmetry axes to assemble the capsid.
- Notable capsid features include a depression at the twofold axes, three protrusions surrounding the threefold axes and a channel with surrounding canyon region at the fivefold axes of symmetry.
- The threefold protrusions are critical for receptor binding and antibody recognition.

AAV as a gene delivery vector

- AAVs can package and express foreign genes in a broad range of tissues.
- The AAVs have specific tissue tropisms, display various transduction efficiencies and have been shown to achieve stable, long-term gene expression.
- AAVs are not associated with any known pathogenicity, making them ideal vectors for gene therapy.

AAV in clinical trials

- Two successful gene therapy trials have involved the use of rAAV2 and rAAV8 to treat Leber's congenital amaurosis and hemophilia B, respectively.
- In late 2012, Glybera® (UniQure) became the first gene therapy treatment approved for clinical use in the western world.

Challenges to AAV gene delivery

- Some cells/tissue types remain refractory to transduction by known AAV serotypes.

- Pre-existing neutralizing antibodies against AAV capsids present in a large portion of the human population can block gene transfer.
- Peptide mapping of antigenic regions and structure determination of AAV capsids complexed with neutralizing antibodies have been used to better understand the immunogenic regions of the capsid.
- Antibodies have been shown to interact with the AAV capsid at common binding regions: around the threefold protrusions, or in the fivefold canyon area.

Chimeric AAV variants

- Chimeric AAV variants can be used to target (or detarget) the virus to specific cell types, or to evade the pre-existing human immune response.
- Directed evolution utilizes randomized AAV *cap* genes to make capsid variants and the variants undergo selective screening for a desired trait (e.g., tissue tropism or evasion of neutralizing antibodies).
- Rational capsid engineering takes advantage of known sequence and structural information to design new chimeric viruses by transferring specific capsid residues from one serotype to another to confer a desired phenotype.

Figure 1. Adeno-associated virus genome organization

Two inverted terminal repeats flank the adeno-associated ssDNA genome, which encodes the *rep*, *cap* and *aap* genes. The *rep* gene encodes four nonstructural proteins: Rep78, Rep68, Rep 52 and Rep40. Three overlapping VP proteins (VP1, VP2 and VP3) are expressed from the *cap* gene. AAP is expressed from a nonconventional start site nested in the *cap* gene.

Figure 2. A simplified adeno-associated virus phylogram

The AAV serotypes are separated into five clades (A–F) and two clonal isolates based on VP1 amino acid sequence. ClustalW2 Phylogeny [111] was used to generate the rooted phylogenetic tree. Distance values are shown for each serotype and indicate the number of substitutions as a proportion of the length of the alignment.

Figure 3. Adeno-associated virus capsid structure

(A) An adeno-associated virus (AAV) VP3 monomer is shown with a conserved core region consisting of eight antiparallel β -sheets (β B– β I) and an α -helix (α A). Loop insertions between the β -sheets vary among the AAV serotypes. Nine VRs (defined in [49]) are present on the capsid surface, and are color coded and denoted with roman numerals (I: purple; II: blue; III: yellow; IV: red; V: gray; VI: hot pink; VII: cyan; VIII: green; IX: brown; β HI loop: tan). (B) Locations of the VRs and viral asymmetric unit (shown in white) on the surface of the AAV capsid. The two-, three- and five-fold symmetry axes make up the border of the viral asymmetric unit, which is defined as the smallest repeating unit on the icosahedral capsid, and 60 of these compose the AAV capsid. It is indicated by a triangle on

the surface of the AAV capsid, encompassing the area from the fivefold pore down to the twofold symmetry axis and outwards to the threefold protrusions.

VR: Variable region.

Figure 4. Adeno-associated virus capsid interactions

(A) An adeno-associated virus capsid is composed of 60 VP monomers. A viral asymmetric unit is depicted (shown in white triangle), as demonstrated in **Figure 3**. (B) A space-filling surface representation of an adeno-associated virus monomer (reference monomer shown in green). (C) A dimer of monomers interacts to form a depression at the twofold axis of symmetry. (D) A trimer in which the interdigitation of three monomers forms the threefold spiky protrusions. (E) A pentamer with five interacting monomers, which has a characteristic central channel that spans the interior and exterior of the capsid.

Figure 5. Models of adeno-associated virus chimeras created by directed evolution

Exterior (left-hand side) and interior (right-hand side) capsid views of (A) adeno-associated virus (AAV)-DJ, (B) AAV-HAE1, (C) AAV-HAE2, (D) AAVM41 and (E) AAV-1829. Capsids are color coded by their contributing serotypes (and in lighter shades if there are multiple contributions from that serotype).

AAV1: purple; AAV2: blue; AAV3: yellow; AAV4: red; AAV5: gray; AAV6: hot pink; AAV7: cyan; AAV8: green; AAV9: brown. The viral asymmetric unit is shown in white (as in Figure 3).

Figure 6. Models of adeno-associated virus chimeras created by rational capsid engineering
 Exterior (left-hand side) and 2D surface roadmap views (right-hand side) of (A) adeno-associated virus (AAV2) Y/F mutants, (B) AAV2 Y/F and T/V quadruple mutant (Y444F + Y500F + Y730F + T491V), (C) AAV2 $\leftrightarrow$ AAV8 loop variant (581–584 and 589–592), (D) AAV2i8, (E) AAV9 variant AAV9.45 and (F) AAV2.5. Capsids are colored gray and altered residues are color coded by their contributing serotypes (and in lighter shades if there are multiple contributions from that serotype). AAV1: purple; AAV2: blue; AAV3: yellow; AAV4: red; AAV5: gray; AAV6: hot pink; AAV7: cyan; AAV8: green; AAV9: brown. The viral asymmetric unit is shown in white on the left and black on the right (as in **Figure 3**).

Table 1

Reported functional roles of adeno-associated virus variable regions.

VR	AAV2 aa	Reported functional role(s)	Ref.
VR-I	260–267	aa 263, 265, determinant of muscle transduction; A20 neutralization; 3C5 binding	[59,63,67,97]
VR-II	326–330	Transduction	[63]
VR-III	380–384	Transduction; A20 neutralization	[63,67,97]
VR-IV	449–467	aa 456–476, liver transduction efficiency determinant; aa 456–568, delayed blood clearance phenotype; 4E4 neutralization	[46,59,98]
VR-V	487–504	aa 498, 503, 504, effect on liver- and muscle-specific transduction; aa 456–568, delayed blood clearance phenotype; C37-B neutralization; 4E4 neutralization; 5H7 neutralization	[46,59,98]
VR-VI	522–538	aa 456–568, delayed blood clearance phenotype; aa 531, airway epithelium determinant	[46,107]
VR-VII	544–557	aa 456–568, delayed blood clearance phenotype; aa 550–568, liver transduction efficiency determinant; A20 neutralization; 3C5 neutralization	[46,59,63,98]
VR-VIII	580–592	aa 592, 595 reduced transduction efficiency in liver; aa 584, 598, airway epithelium determinant; aa 585–590, ability to traverse blood vasculature phenotype; aa 602, effect on liver- and muscle-specific transduction; aa 581–584 and 589–592, effect on liver and heart-specific transduction; ADK8 neutralization; C37-B neutralization; 5H7 neutralization	[44,46,59,60,96,98,107]
VR-IX	703–711	aa 699–735, determinant of heart tropism; determinant of improved melanoma tropism in AAV-1829; aa 706, mutants exhibited altered tropism; aa 705, 708, 716, determinant of muscle transduction; A20 neutralization; 3C5 binding	[59,63,91,97]

aa: Amino acid; AAV: Adeno-associated virus; VR: Variable region.