

Article

A Rationally Designed AAV9-DM Capsid with Minimal Liver Tropism

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Highlights

What are the main findings?

- The AAV9-DM capsid results in ~127 fold lower liver transduction as compared to AAV9 in mice.
- The AAV9-DM capsid exhibits a biodistribution similar to AAV9 and results in durable transgene expression in mice.

What are the implications of the main findings?

- AAV9-DM can be used to target extrahepatic tissue, especially the CNS, while avoiding liver transduction.
- AAV9-DM may allow for the use of lower doses when targeting the CNS via peripheral injection, resulting in reduced immune responses in the clinic.

Abstract

Adeno-associated viral vectors (AAV) are the leading gene therapy in the clinic. AAV9 has been of particular interest due to its wide tropism for multiple tissue types as well as being able to cross the blood-brain barrier and transduce central nervous system tissues. However, effectively and safely targeting extrahepatic tissue following the systemic administration of AAV9 remains a challenge due to high rates of liver transduction and liver toxicity. Thus, a crucial first step in developing a safe AAV9-based vector is to reduce liver targeting. Here we utilized rational design techniques to make five point mutations in the AAV9 capsid. In doing so, we developed a novel AAV9 variant, AAV9-DM, that is characterized by reduced liver tropism as compared to AAV9 and other liver de-targeted AAV9 mutants. We show that AAV9-DM is effective at transducing cells in vivo, resulting in robust transgene expression over a 9-week period. Importantly, the AAV9-DM capsid maintains the ability to transduce non-hepatic tissues with a biodistribution similar to AAV9. This new mutant represents a novel AAV capsid that may be the basis for developing safer therapeutics to target extrahepatic tissue while reducing adverse side effects related to liver transduction.

Keywords: adeno-associated virus; gene therapy; liver de-targeted; rational design; capsid engineering



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1. Introduction

Adeno-associated virus (AAV) is a small, non-pathogenic parvovirus that requires co-infection with a helper virus such as adenovirus for optimal gene expression and replication [1]. Without a helper virus, AAV genomes remain inactive, establish a latent

infection, generally in the episomal form, and do not replicate [1–3]. The non-pathogenic nature, minimal integration, and low immunogenicity associated with AAV led to the development of recombinant AAV vectors [4,5]. AAV vectors have been used extensively to treat a wide variety of human diseases. In the clinic, over 300 phase I, II, and III clinical trials have used or are soon to use AAV technology. This has led to the U.S. Food and Drug Administration (FDA) granting approval for eight AAV drugs with six currently on the market: Luxturna for the treatment of retinal dystrophy [6], Zolgensma for the treatment of spinal muscular atrophy (SMA) [7], Hemgenix for the treatment of hemophilia B [8], Elevidys for the treatment of Duchenne Muscular Dystrophy (DMD) [9], Roctavian for the treatment of hemophilia A [10], and Kebilidi for the treatment of aromatic L-amino acid decarboxylase deficiency [11].

Despite clinical achievements, it has become apparent that current first-generation AAV therapies are not optimal. In many instances, systemic AAV administration is preferable due to low invasiveness and broad biodistribution. However, systemic AAV administration often leads to relatively low therapeutic indices in extrahepatic tissues due to high levels of liver transduction [12]. Further, the high doses being used in clinics have been shown to result in serious adverse events (SAEs) and have culminated in at least 21 patient deaths [13–15] (Clinicaltrials.gov).

AAV serotype 9 (AAV9) is appealing due to its broad tropism, ability to cross the blood-brain barrier (BBB), and ability to transduce central nervous system (CNS) cells following systemic injection. Thus, AAV9 has been utilized extensively in the clinic to treat multiple diseases and has become the ‘gold standard’ AAV serotype for targeting the CNS via the BBB in the clinic. Unfortunately, clinical results using AAV9 have been mixed at best. While Zolgensma, an AAV9-based therapy, gained FDA approval, its effectiveness is less than optimal. Zolgensma has resulted in transaminitis in up to 90% of patients and has led to liver failure in some patients [16–18]. In other trials, five of the eight AAV therapies that resulted in patient death used AAV9 and ~50% of those deaths resulted from acute liver toxicity (Clinicaltrials.gov). Thus, for AAV9-based therapies to reach their full potential in the clinic, a crucial first step is to develop new capsids that avoid liver transduction without rendering the vectors unable to target extrahepatic tissue. These liver de-targeted vectors will provide the basis for developing new, ‘targeted’ AAV vectors that have improved transduction rates in various extrahepatic tissues while avoiding liver toxicity.

In this work we set out to develop an AAV9-based capsid that results in minimal liver transduction levels while maintaining the ability to transduce skeletal muscle, cardiac tissue, the brain, and spinal cord. Previously, we showed the importance of residues 501, 505, and 706 in determining liver tropism in multiple AAV capsids (AAVx-HSC) [19]. Others have shown that residues 527 and 533 play an important role in AAV9 liver tropism in neonates [20]. Here we developed and tested the ability of a highly modified AAV9 capsid containing point mutations at residues 501, 505, 527, 533, and 706 (AAV9-DM) to avoid liver transduction while maintaining extrahepatic tissue transduction. Our *in vivo* bioluminescence studies showed that the AAV9-DM capsid resulted in durable transgene expression spanning a 9-week period. Further, quantification of genome copies and mRNA levels showed that the AAV9-DM vector maintained a biodistribution profile similar to AAV9, albeit at reduced frequencies.

2. Materials and Methods

2.1. Animals and AAV Administration

Eight-week-old female C57BL/6 mice, purchased from the Jackson Laboratory, were used. Animals were housed in a pathogen-free animal vivarium with access to food, *ad libitum*, on a 14/10 h on/off light cycle. Animals were allowed to acclimate in the vivarium

for one week prior to experiments. Animals were injected with 1×10^{11} vg/animal (in sterile saline (Aspen Veterinary Resources, Liberty, MO, USA)) via the tail vein. All animal studies were carried out in accordance with and approved by the University of Florida Institutional Animal Care and Use Committee (IACUC).

2.2. Vector Production and Packaging Efficiency

A recombinant single-strand expression cassette containing firefly luciferase (Fluc) transgene under control of the chicken beta-actin (CBA) promoter was packaged into AAV9, AAV9-16, AAV9-HR, or AAV9-DM capsids, as previously described [21,22]. Virus was produced in human embryonic kidney (HEK 293) cells (ATCC Manassas, VA, USA) below passage 30 using the three-plasmid system [23]. Briefly, confluent cells were co-transfected with three plasmids: one containing the promoter and transgene, one containing the Rep gene and viral capsid sequence, and pHelper. Seventy-two h later, cells and media were harvested, and the virus was purified via an iodixanol gradient [24]. Viral titers were determined via quantitative polymerase chain reaction (qPCR) using primers specific to the transgene [25]. All viruses were purified into 1 mL aliquots and yields were compared.

2.3. Bioluminescence

In vivo grade D-luciferin (Promega Corporation, Madison, WI, USA) was diluted in sterile PBS and stored at -20°C until use. Mice were anesthetized via 3% isoflurane (Patterson Veterinary Supply, Loveland, CO, USA) vaporized in oxygen, and 300 μL of D-luciferin solution was injected subcutaneously (subq). Ten minutes later, animals were placed in a Xenogen IVIS Spectrum in vivo imager (PerkinElmer, Waltham, MA, USA), maintained on 2% isoflurane vaporized in oxygen, and radiance (luminescence) was measured as photons/sec/cm²/sr (number of photons emitted in a certain area per second at a specific solid angle).

2.4. qPCR

Quantitative polymerase chain reaction was used to quantify vector genomes in sample tissues using standard protocols. Briefly, following image studies at the 9-week timepoint, animals were euthanized, and tissues were harvested, snap frozen in liquid nitrogen, and stored at -80°C until processed. To extract DNA, tissue was homogenized in ATL tissue lysis buffer (Qiagen, Hilden, Germany) using sterile, DNase/RNase-free beads and a bead basher. DNA was then purified using a 2-step process: (1) chloroform (Thermo Scientific, Carlsbad, CA, USA); (2) a DNeasy kit (Qiagen, Hilden, Germany). SYBR Green master mix (Fisher Scientific, Carlsbad, CA, USA) and transgene-specific primers (forward: ATGGACAGCAAGACTGACTAC; reverse: AGCTCCTCCTCAAACCTATAC) (Eurofins Genomics, Louisville, KY, USA) were used to quantify vector genomes in sample. A standard curve consisting of linearized Fluc plasmid was used to quantify the vector genomes in each sample. PCR was performed for 40 cycles using a CFX96 Optics Module Thermal Cycler (Bio Rad, Hercules, CA, USA).

2.5. RT-qPCR

Following the final imaging at 9 weeks post-vector administration, animals were euthanized, their tissue was harvested and immediately frozen in liquid nitrogen, and their RNA was extracted, converted to cDNA, and quantified, as previously described [26,27]. Briefly, to extract RNA, tissue was homogenized in Trizol (Invitrogen, Waltham, MA, USA) and sterile RNase/DNase-free beads in a bead basher. RNA was then purified using a RNeasy kit (Qiagen, Hilden, Germany), integrity was confirmed by gel electrophoresis looking at the 18s and 28s band ratios, and RNA was converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, WA, USA). TaqMan

gene expression assays (Life Technologies, Waltham, WA, USA) were utilized to determine relative gene expression ratios via a CFX96 Optics Module Thermal Cycler (Bio Rad, Hercules, CA, USA) (forward primer: AGTGGTGCTGATGTATAGGTTTGAG; reverse: AGGGCAGACTGGATCTTGAGT). Transcription initiation factor TFIID subunit 9 (Taf9b) was used as a housekeeping gene. The $2^{-\Delta\Delta CT}$ method was used to identify differences in relative gene expression ratios.

2.6. Statistical Analysis

In this work all data are presented as mean $\pm$ SEM. To identify differences amongst groups, a multiple comparison, Kruskal–Wallis with a Dunn’s post hoc test was performed to compare the mean of each group to the mean of every other group. All data were determined to be statistically significant at a $p \leq 0.05$. All statistical analyses were performed using GraphPad Prism software (Version 10.6.1, San Diego, CA, USA).

3. Results

3.1. Rational Design of AAV9-DM

AAVHSC16 is a naturally occurring, clade F AAV serotype that exhibits reduced liver transduction while maintaining tropism for extrahepatic tissue in murine and non-human primate models, as compared to other clade F members [28]. In previous work, we confirmed that residues 501, 505, and 706 are responsible for reduced liver transduction and can be used to reduce liver transduction rates in non-clade F AAV serotypes [19]. AAV9-HR was created by mutating residues 527 and 533 in the wt AAV9 capsid, resulting in a capsid with reduced liver transduction which maintained the wt capsid’s innate extrahepatic tissue tropism [20]. We created the AAV9-16 capsid by mutating phenylalanine at position 501 to isoleucine, glycine at position 505 to arginine, and tyrosine at position 706 to cysteine in the AAV9 capsid. Here, we sought to determine if combining all five of these point mutations would have a synergistic effect on liver de-targeting while maintaining the ability to transduce extrahepatic tissue. To create the AAV9-DM capsid, we combined the 501, 505, 706, 527, and 533 mutations in one AAV9 capsid (Figure 1A).

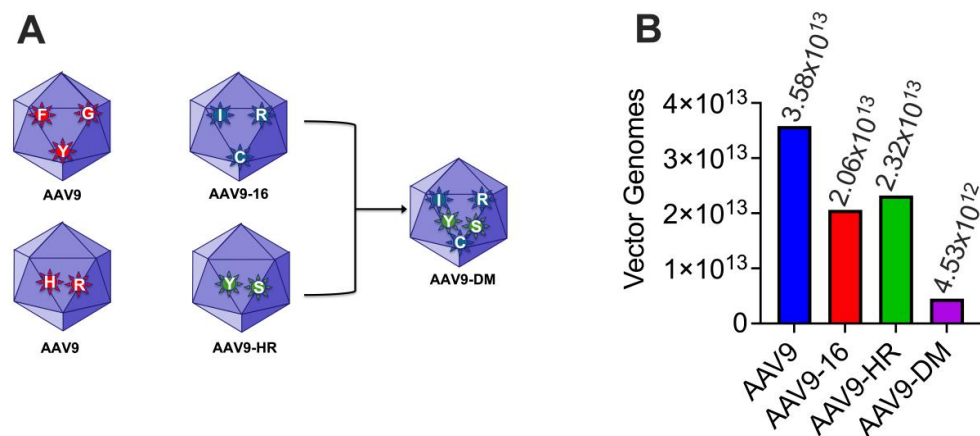


Figure 1. Development of AAV9-DM capsid. (A) Point mutations at positions 501, 505, and 706 from the AAV9-16 capsid and at positions 527 and 533 from AAV9-HR capsid were combined to create the AAV9-DM capsid. (B) Packaging efficiencies were determined for AAV9, AAV9-16, AAV9-HR, and AAV9-DM. All mutant capsids are packaged less efficiently than AAV9, with the AAV9-DM capsid being the least efficient. F = Phenylalanine, G = Glycine, Y = Tyrosine, I = Isoleucine, R = Arginine, C = Cysteine, H = Histidine, S = Serine.

3.2. Packaging Efficiencies

Rationally designed AAV capsids contain mutations within the capsid sequence to elicit a desired functional effect. While rational design is a common technique to alter the tropism of AAV capsids, it has the potential to adversely affect the wild-type (wt) capsid structure and greatly reduce or prevent AAV genomes from being packaged into the capsid. Viral vector yield studies assessing side-by-side packaging efficiencies compared to wt capsids are used to determine structural compatibility. Here, we packaged single-strand (ss) AAV genomes encoding firefly luciferase (Fluc) under the control of the ubiquitous chicken beta-actin (CBA) promoter from 1.6×10^9 human embryonic kidney 293 (HEK293) cells into AAV9, AAV9-16, AAV9-HR, and AAV9-DM capsids. AAV9 yields were in line with the general field consensus at 3.58×10^{13} vector genomes (vgs) (Figure 1B). We found both AAV9-16 and AAV9-HR to result in slightly lower yields than AAV9 at $\sim 2.0 \times 10^{13}$ vgs (Figure 1B). However, the reduction in packaging efficiency was more pronounced in the AAV9-DM, which yielded 4.5×10^{12} vgs (Figure 1B). These results corroborate the idea that capsid modifications can result in reduced packaging efficiencies, with a correlation existing between the level of capsid modification and the reduction in efficiency.

3.3. AAV9-DM Capsid Results in Liver De-Targeting and Durable Transgene Expression

Initial studies were performed to evaluate transgene expression levels and whether the AAV9-DM mutant resulted in reduced transgene expression in the liver as compared to AAV9, AAV9-16, and AAV9-HR. To determine this, 1×10^{11} vgs/animal of AAV9, AAV9-16, AAV9-HR, or AAV9-DM capsids containing a Fluc transgene were injected into C57BL6/J mice via the tail vein. Following the administration of luciferin, we evaluated the average p/sec/cm²/sr (average radiance, AR) emitted from the ventral side of the animal using a Xenogen IVIS Spectrum in vivo imager. Animals were imaged and radiance quantified at 3, 4, 5, 6, 7, and 9 weeks post-AAV administration (Figure 2A). The bioluminescence emitted from these animals occurred over a wide range. Based on this, we separated animals into three different groups according to AR. AAV9 was placed in the high group and reached a peak AR of 7.3×10^7 , AAV9-16 and AAV9-HR were in the medium group and reached a peak AR of 6.6×10^6 and 3.5×10^6 , respectively, and AAV9-DM was in the low group and reached a peak AR of 5.8×10^5 (Figure 2). Our results show that AAV9 resulted in robust transgene expression (AR) in the liver (Figure 2B). In extrahepatic tissue, widespread expression occurred at levels lower than the liver at each timepoint tested (Figure 2B). These findings are in line with previous work that showed that systemically administered AAV9 results in robust transgene expression in the liver and lower, widespread expression in extrahepatic tissue up to 56 days post-AAV administration [29]. The AAV9-16, AAV9-HR, and AAV9-DM capsids all resulted in similar expression patterns as AAV9, albeit at reduced levels, at all of the timepoints tested (Figure 2B). The quantification of radiance from the liver shows that AAV9-16, AAV9-HR, and AAV9-DM result in a significantly decreased expression as compared to AAV9 at each of the timepoints tested (Figure 3A). Importantly, the AAV9-DM capsid results in significantly reduced transgene expression in the liver as compared to either AAV9-16 or AAV9-HR at all timepoints tested (Figure 3A). Furthermore, all vectors tested resulted in a detectable expression over the entire 9-week period (Figure 3B). The AAV9-16 and AAV9-HR mutants result in a stable expression, with no differences occurring over the 9-week time course (Figure 3B). Interestingly, transgene expression from AAV9 was stable over a 7-week period but was found to be significantly reduced at week 9 as compared to week 3 (Figure 3B). Finally, the AAV9-DM mutant resulted in significantly reduced transgene expression levels at 6 and 9 weeks as compared to week 3, though no differences were seen at other timepoints (Figure 3B). While we found significant differences in transgene expression levels as compared to week three, it

is important to note that, after AAV administration, early peaks in transgene expression followed by stabilization are not uncommon [30–34]. Taken together, this data suggests that mutations in AAV9-DM work synergistically to avoid liver expression more efficiently than AAV9, AAV9-16, and AAV9-HR, while maintaining the ability to target extrahepatic tissues.

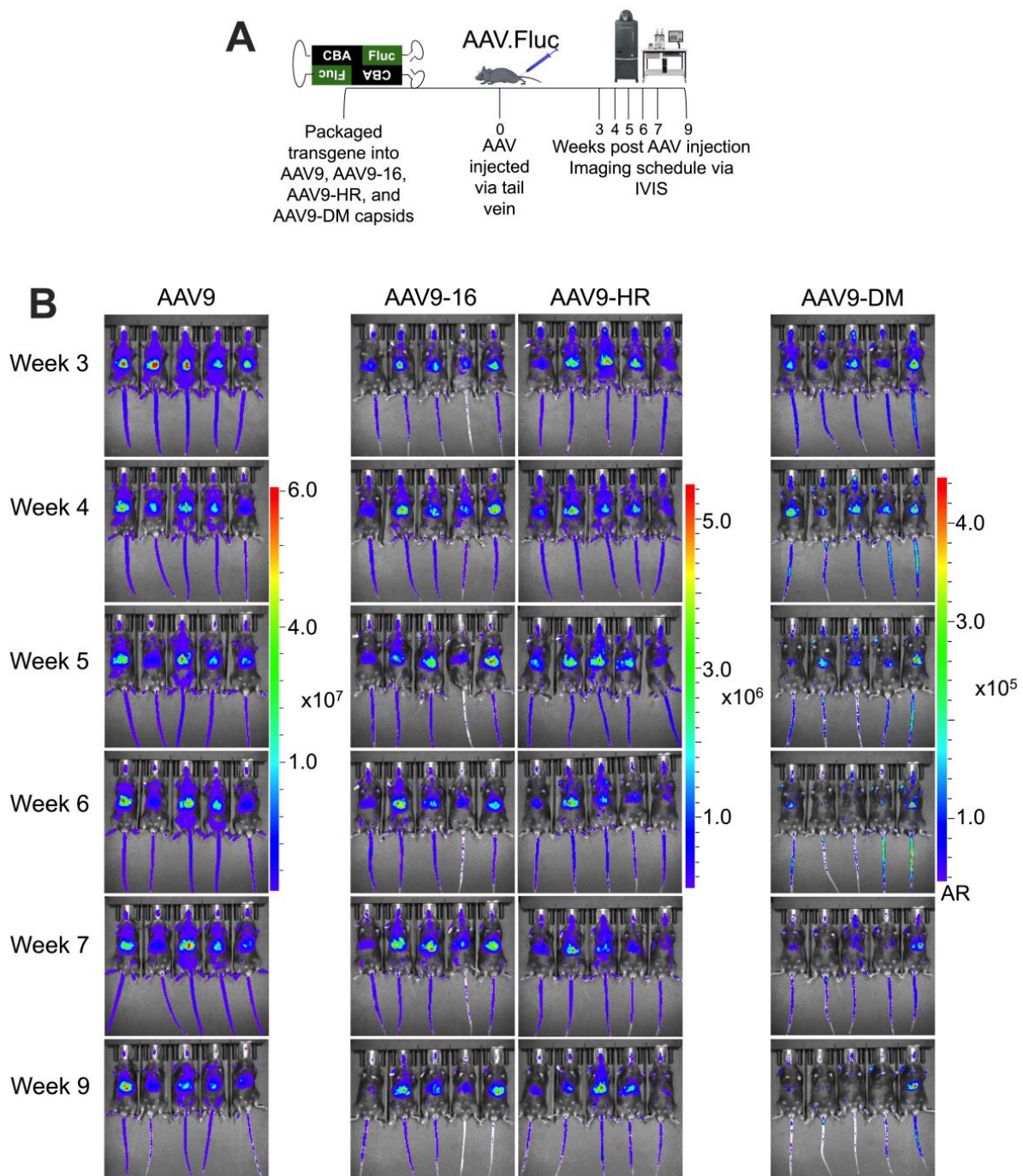


Figure 2. In vivo imaging of bioluminescence following i.v. administration of AAV. **(A)** Single-strand AAV containing firefly luciferase under the control of a CBA promoter was packaged into AAV9, AAV9-16, AAV9-HR, and AAV9-DM. The CBA promoter allows transgene expression to be visualized in all transduced tissues. **(B)** Bioluminescence studies were performed at 3, 4, 5, 6, 7, and 9 weeks post-AAV injection. Animals were separated into 3 groups (high, medium, and low) based on AR of transgene expression. AAV9 results in robust transgene expression in various tissues. AAV9-16, AAV9-HR, and AAV9-DM result in a similar biodistribution as AAV9, albeit at reduced levels.

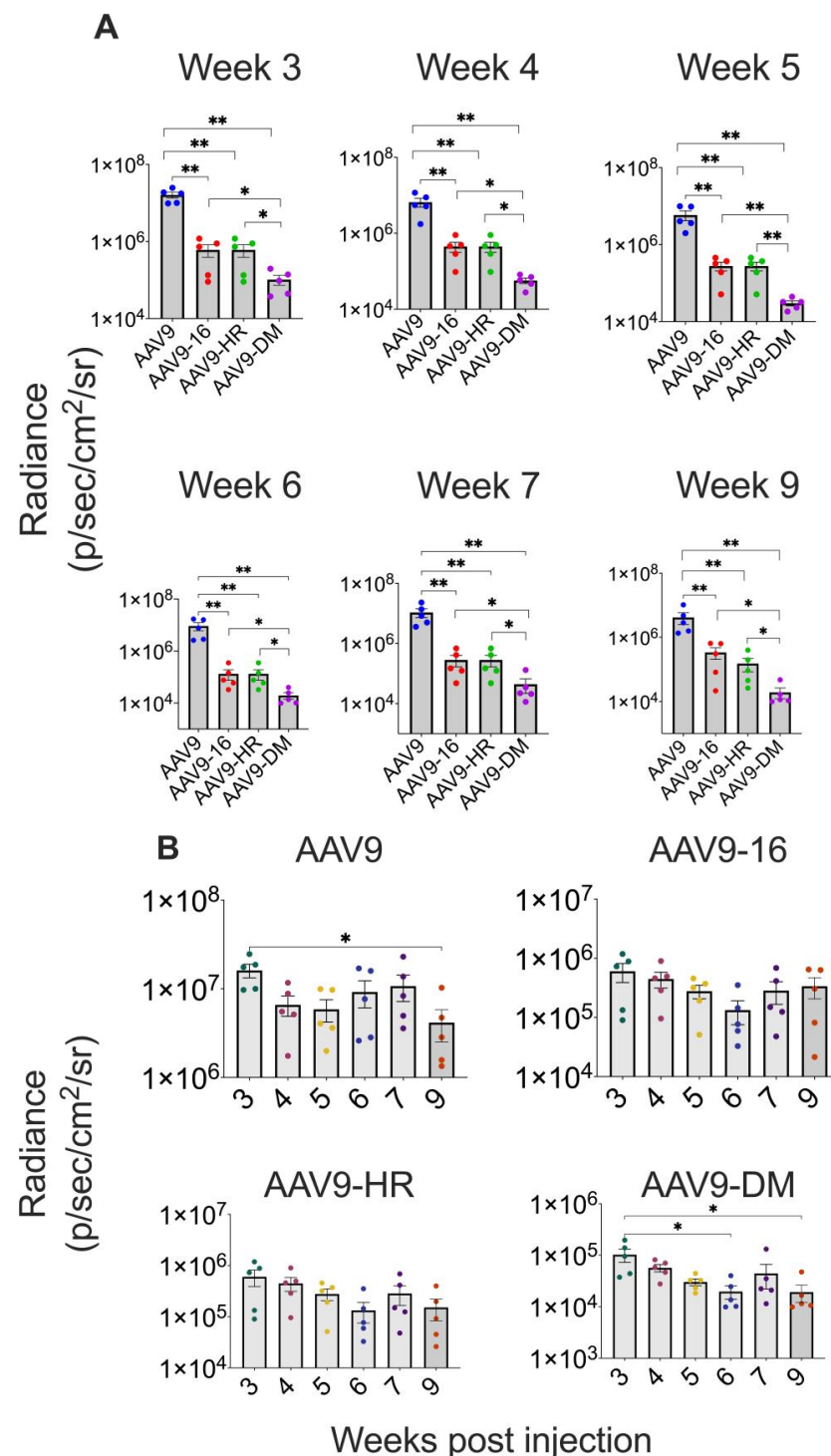


Figure 3. AAV9-DM results in reduced transgene expression in the liver of C57BL/6 mice. **(A)** AAV9-16, AAV9-HR, and AAV9-DM capsids all resulted in significantly reduced transgene expression (AR) in the liver as compared to AAV9. AAV9-DM resulted in significantly reduced transgene expression (AR) in the liver as compared to AAV9-16 and AAV9-HR. **(B)** Transgene expression, as measured by AR, was durable and persisted over the course of the nine-week period. Data represented as mean $\pm$ SEM, $n = 5$, * $p \leq 0.05$, ** $p \leq 0.01$. All other comparisons did not reach significance.

3.4. Evaluation of Vector Genome Biodistribution in Different Tissues

Following the 9-week imaging timepoint, the liver, heart, gastrocnemius (GA), brain, and spinal cord were harvested, and a portion of each tissue was used for quantitative polymerase chain reaction (qPCR) to evaluate the vector genome copy biodistribution in

different tissues for each vector. Results were normalized to the μg of total DNA analyzed and were presented as $\text{vg}/\mu\text{g}$ total DNA. The AAV9 vector resulted in robust vector genome copies in all tissues tested (Figure 4A–E). AAV9 resulted in similar genome copies in the liver and heart, consistent with previous findings that show similar AAV9 vector genome copies in the liver and heart, while lower copy numbers are found in the GA, brain, and spinal cord [20,29,35–37]. The AAV9-16, AAV9-HR, and AAV9-DM mutants resulted in significantly lower vector genome copies in the liver as compared to AAV9 (Figure 4A,D,E). AAV9-DM resulted in a ~ 127 fold reduction in vector genome copies as compared to AAV9, which is the most significant reduction in any variant tested (Figure 4A). In the heart, AAV9-HR and AAV9-DM resulted in reduced vector genome copies, while AAV9-16 was not significantly different from the levels achieved with AAV9 (Figure 4B). In the GA, AAV9-DM resulted in vector genome copies consistent with levels achieved from AAV9, while the AAV9-16 and AAV9-HR vectors resulted in levels below the detection limit of the assay (Figure 4C). In the brain, AAV9-HR resulted in the largest reduction in vector genome copies as compared to AAV9, with AAV9-16 and AAV9-DM being reduced to a lesser degree (Figure 4D). Finally, in the spinal cord, AAV9-HR led to a significant reduction in vector genome copies, while AAV9-16 and AAV9-DM were equivalent to what resulted from AAV9 (Figure 4E).

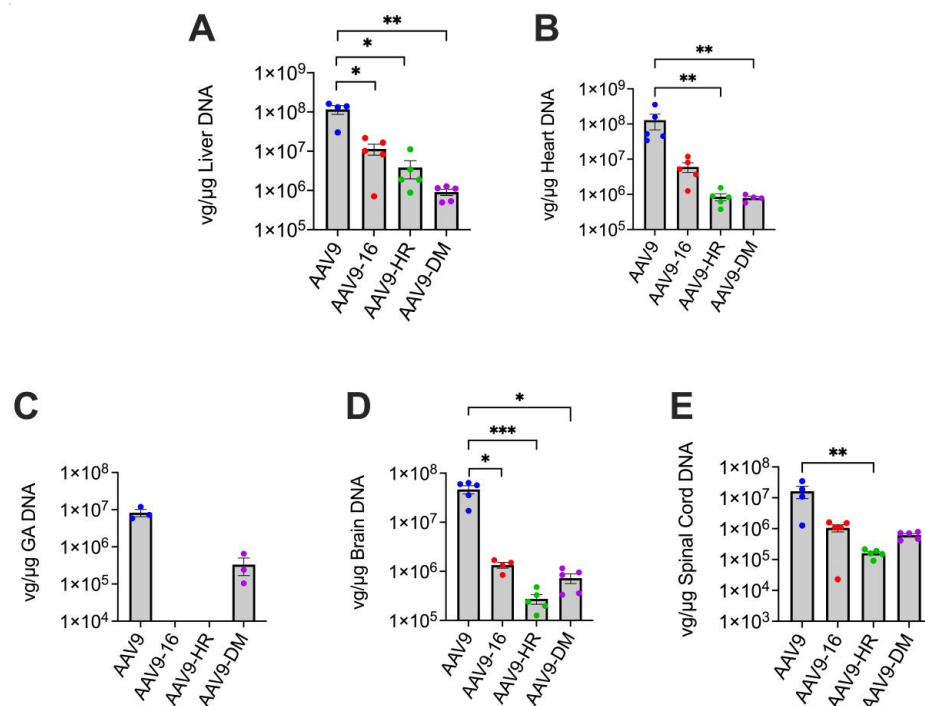


Figure 4. Evaluation of vector genome biodistribution from AAV9, AAV9-16, AAV9-HR, and AAV9-DM in different tissues in C57BL/6 mice. (A) AAV9-16, AAV9-HR, and AAV9-DM resulted in reduced vector genome copies in the liver as compared to AAV9. (B) AAV9-HR and AAV9-DM resulted in reduced vector genome copies in the heart, while AAV9-16 resulted in equivalent vector genome copies as AAV9. (C) AAV9-DM was the only variant with detectable vector genome copies in the GA, and they were equivalent to AAV9. AAV9-16 and AAV9-HR were below assay detection limits. (D) AAV9-16, AAV9-HR, and AAV9-DM resulted in reduced vector genome copies in the brain, with AAV9-HR having the most significant decrease as compared to AAV9. (E) In the spinal cord, AAV9-HR resulted in significantly reduced vector genome copies, while AAV9-16 and AAV9-DM were equivalent to AAV9. Data represented as mean $\pm$ SEM, $n = 3$ –5, * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. All other comparisons did not reach significance.

3.5. Evaluation of Fluc Relative Gene Expression Levels in Extrahepatic Tissues

To evaluate tissue-specific differences in transgene expression levels associated with the different vectors, we performed a relative gene expression of Fluc via RT-qPCR in various tissues. In the liver, AAV9-16, AAV9-HR, and AAV9-DM all resulted in a significantly reduced relative gene expression as compared to AAV9 (Figure 5A). Importantly, AAV9-DM resulted in the greatest reduction in the gene expression ratio in the liver (Figure 5A). This is in line with the results from the vector genome copy study. In heart tissue, AAV9-DM capsids resulted in significantly reduced relative gene expression levels as compared to AAV9 (Figure 5B). AAV9-16 and AAV9-HR were equivalent to AAV9 in the heart (Figure 5B). Interestingly, in the GA, AAV9-HR resulted in significantly increased relative gene expression levels as compared to AAV9 (Figure 5C). AAV9-16 and AAV9-DM did not result in a significant gene expression ratio change in the GA (Figure 5C). In the brain and spinal cord, all capsids resulted in equivalent relative gene expression levels (Figure 5D,E). While the gene expression ratios are largely in line with vector genome copy data, some discrepancies occurred. In particular, AAV9-HR had higher relative gene expression ratios in the heart and GA from reduced genome copies. Similarly, all of the mutant capsids resulted in equivalent gene expression ratios to AAV9 in the brain and spinal cord. This suggests that the capsid proteins may play a role in tissue-specific transgene expression.

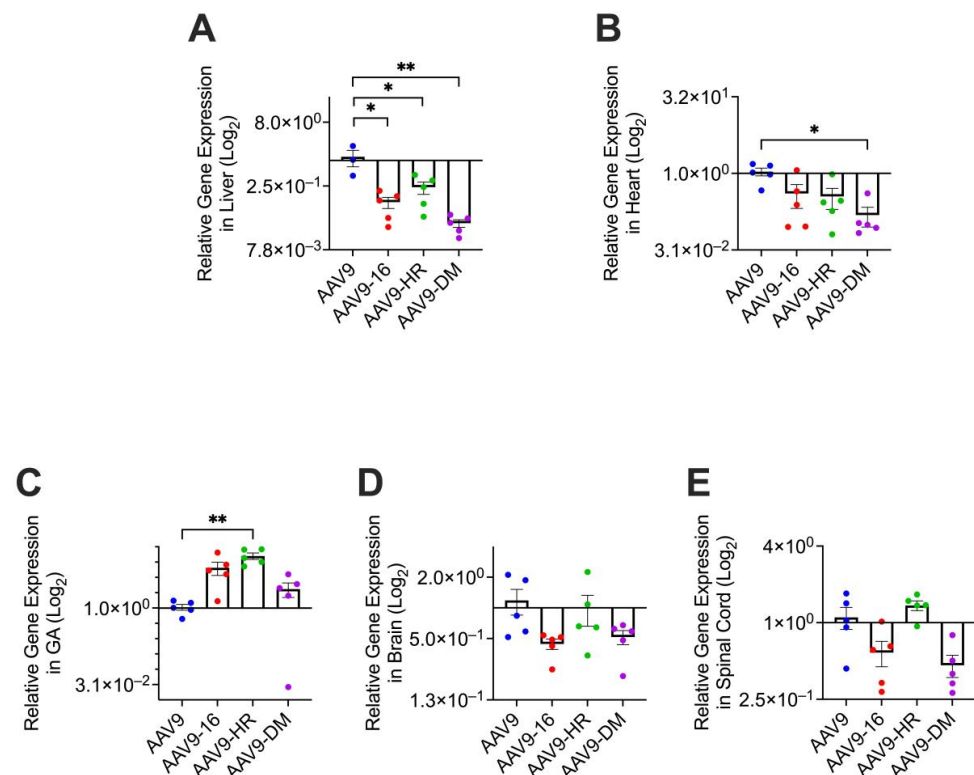


Figure 5. Relative gene expression ratios from AAV9, AAV9-16, AAV9-HR, and AAV9-DM in different tissues. (A) AAV9-16, AAV9-HR, and AAV9-DM resulted in significantly reduced relative gene expression ratios in the liver as compared to AAV9, with AAV9-DM being the most reduced. (B) AAV9-16 and AAV9-HR resulted in similar relative gene expression ratios as compared to AAV9 in the heart. AAV9-DM resulted in reduced relative gene expression ratios in the heart as compared to AAV9. (C) AAV9-HR resulted in a significantly higher relative gene expression ratio as compared to AAV9 in the GA. Relative gene expression ratios from AAV9-16 and AAV9-DM were unchanged as compared to AAV9. (D) AAV9-16, AAV9-HR, and AAV9-DM resulted in relative gene expression ratio levels equal to AAV9 in the brain. (E) AAV9-16, AAV9-HR, and AAV9-DM resulted in unchanged relative gene expression ratios as compared to AAV9 in the spinal cord. Data represented as mean $\pm$ SEM, $n = 3$ –5, * $p \leq 0.05$, and ** $p \leq 0.01$. All other comparisons did not reach significance.

4. Discussion

It is becoming clear that current, first-generation AAV vectors are not optimal for use in humans [38–45] and result in low therapeutic indices. To reach levels required for therapeutic efficacy, high doses of AAV are being administered to patients [45]. This is leading to catastrophic consequences, which are clearly supported by clinical trial results. In an XLMTM trial, three patients died after receiving 3×10^{14} vg/kg of a first-generation AAVrh10 vector; in an MPS-IIIa trial, one patient died after receiving 7×10^{12} vg/kg of an AAVrh10 vector; and in a DMD trial, two patients died after receiving 1×10^{14} vg/kg of an AAVrh74 vector [46]. AAV9 therapies have fared even worse than other vectors. Various AAV9-based therapies have led to eight patient deaths in an SMA trial after receiving 1.1×10^{14} vg/kg [17,42,47,48], two patient deaths in a DMD trial after receiving 2×10^{14} vg/kg [49,50], one patient death in a separate DMD trial after receiving 1×10^{14} vg/kg [51], one patient death in a Rett Syndrome trial after receiving 3×10^{15} vg (total) [52], and one patient death in a Danon disease trial after receiving 6.7×10^{13} vg/kg (though it is unclear if the death was due to AAV9, the immunosuppressant drug administered, or both) [53,54]. Shockingly, the mid-point of these doses (1.0×10^{14} vg/kg) results in 7 quadrillion vector genomes being administered to a patient. In an ‘average’ 70 kg patient, this equates to there being ~234 times the number of vector genomes as cells in the body [55]. When administering hundreds of times more vector genomes to patients than cells in the body, SAEs and deaths should not come as a surprise. At doses in excess of 1×10^{14} vg/kg, systemic AAV administration leads to viremia at levels that have never been seen in natural infections [39]. We believe that for AAV therapies to continue to move forward, the trend of administering high-vector doses must be abandoned in favor of developing efficient AAV vectors that are effective at lower, safer doses.

Where AAVs are being utilized to target extrahepatic tissue, we believe that the propensity of AAV to transduce liver tissue is one of the largest hurdles that must be overcome. In the case of AAV9, de-targeting the vector is a crucial first step in developing the next generation of safe, effective vectors for targeting extrahepatic tissues. To address this, we previously developed AAV3B-16, rh74-16, AAV8-16, and AAV6-16 vectors that showed significant reductions in liver transduction as compared to parental wt vectors [19]. Unfortunately, none of those capsids are efficient at crossing the BBB, precluding them from use in treating CNS diseases.

AAV9-HR is a result of capsid modifications made to AAV9 to produce a new serotype with reduced liver transduction [20]. AAV9-HR results in reduced transduction rates and transgene expression in the liver, though to a lesser extent than what is seen from AAVx-16 variants [19,20,28]. Importantly, AAV9-HR results in CNS transduction and expression rates similar to what is achieved by AAV9 [20]. Here, we tested the theory that combining the AAVx-16 and AAV9-HR mutations would have a synergistic effect in reducing liver transduction rates while allowing for the transduction of extrahepatic tissues. In the liver, AAV9-DM resulted in the most significant reduction in vector genome copies.

Interestingly, vector genome copies resulting from AAV9-DM in other tissues are not consistent with either parent capsid. In the heart, AAV9-DM results in vector genome copies similar to AAV9-HR, which are lower than AAV9-16. Conversely, in the brain and spinal cord, AAV9-DM results in vector genome copy numbers similar to AAV9-16, which are higher than what is present from AAV9-HR. This dichotomy underscores the importance of careful capsid selection based on the target tissue. Of the capsids investigated here, we found that AAV9-16 may be the best option for targeting the heart, while AAV9-DM may be more appropriate for targeting the CNS with minimal off-target transduction.

These findings are supported by relative gene expression ratio studies that show that AAV9-DM resulted in the lowest expression ratios in the liver and heart, while being

equivalent to AAV9 in all other tissues. Interestingly, results from vector genome copy biodistribution and relative gene expression ratios did not agree in the GA, brain, and spinal cord. In the GA, AAV9-16 and AAV9-HR did not result in any detectable vector genome copies, whereas these two both resulted in detectable gene expression, with AAV9-HR being significantly higher than AAV9. In the brain, all mutated capsids resulted in significantly lower vector genome copies as compared to AAV9, though relative expression levels were consistent amongst all capsids investigated. While these findings seem counterintuitive, they are in line with previous results that have suggested the AAV capsid may play a role in transcription [56,57] and more recent results that have shown the AAV9 capsid to interact with promoters and alter vector genome expression levels as well as tropism in rats and non-human primates [58–61]. Whether the mutated capsids are modulating transgene transcription and the mechanisms through which this is occurring are yet to be determined, and this represents an avenue that requires further investigation.

In sum, we have developed a new AAV9-DM capsid that possesses reduced liver tropism and reduced heart tropism, while minimally affecting CNS tropism as compared to AAV9. This is a critical first step in developing the next generation of AAV vectors. AAV9-DM represents an attractive option for developing treatments where extrahepatic tissues are being targeted, especially those treatments targeting the CNS. Moving forward, the AAV9-DM capsid can be further engineered to develop new AAV capsids that are administered at low, safe doses for effectively targeting specific tissues at high rates and resulting in reduced liver toxicities in the clinic.

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Conflicts of Interest: G.D.K. is an inventor on a pending patent related to this work.

Abbreviations

The following abbreviations are used in this manuscript:

AAV	Adeno-associated Virus
FDA	Food and Drug Administration
SMA	Spinal Muscular Atrophy
DMD	Duchenne Muscular Dystrophy
SAEs	Severe Adverse Side-effects
BBB	Blood-Brain Barrier
CNS	Central Nervous System
Wt	Wild-type
Ss	Single-strand
Fluc	Firefly Luciferase
CBA	Chicken Beta-actin
HEK293	Human Embryonic Kidney 293 Cells
Vgs	Vector Genomes

i.p.	Intraperitoneal
GA	Gastrocnemius
qPCR	Quantitative Polymerase Chain Reaction
XLMTM	X-Linked Myotubular Myopathy
MPS III	Mucopolysaccharidosis Type III

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