Glycan binding avidity determines the systemic fate of adeno-associated virus 9

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Glycans are key determinants of host range and transmissibility in several pathogens. In the case of adeno-associated viruses (AAV), different carbohydrates serve as cellular receptors in vitro; however, their contributions in vivo are less clear. A particularly interesting example is adeno-associated virus serotype 9 (AAV9), which displays systemic tropism in mice, despite low endogenous levels of its primary receptor (galactose) in murine tissues. To understand this further, we studied the effect of modulating glycan binding avidity on the systemic fate of AAV9 in mice. Intravenous administration of recombinant sialidase increased tissue levels of terminally galactosylated glycans in several murine tissues. These conditions altered the systemic tropism of AAV9 into a hepatotropic phenotype, characterized by markedly increased sequestration within the liver sinusoidal endothelium and Kupffer cells. In contrast, an AAV9 mutant with decreased glycan binding avidity displayed a liver-detargeted phenotype. Altering glycan binding avidity also profoundly affected AAV9 persistence in blood circulation. Our results support the notion that high glycan receptor binding avidity appears to impart increased liver tropism, while decreased avidity favors systemic spread of AAV vectors. These findings may not only help predict species-specific differences in transduction profile for AAV9 on the basis of tissue glycosylation profile, but also provide a general approach to tailor AAV vectors for systemic or hepatic gene transfer by reengineering capsid-glycan interactions.
INTRODUCTION

The cell surface glycocalyx contains many glycans terminated with sialic acid moieties. Variability in the extent, type and linkage of sialylation between different hosts is exploited by several pathogens during infection (21, 33). Sialo/asialoglycans have also been implicated as cell surface receptors for different strains of adeno-associated virus (AAV) \textit{in vitro} (1, 23). The latter are non-pathogenic, helper-dependent parvoviruses, currently being evaluated in phase I-III clinical trials as vectors for gene therapy (20). Over 150 different strains have been isolated from human and primate tissues till date and these AAV isolates display striking differences in capsid structure, receptor usage and tissue tropism within different hosts (1, 4, 31). A better understanding of the mechanisms underlying AAV tropism in different hosts could help guide the selection of preclinical animal models for evaluating AAV vectors and possibly predict outcomes in human gene therapy clinical trials.

Glycan receptors for different AAV serotypes include heparan sulfate for AAV2 (30), N-linked α2,3-/α2,6-sialic acid for AAV1/AAV6 (36), N-linked α2,3-sialic acid for AAV5 (35), O-linked α2,3-sialic acid for AAV4 (12) and β1,4-galactose for AAV9 (6, 28). In addition, various co-receptors such as integrins and growth factor receptors have been implicated in AAV infection \textit{in vitro} (1, 23). Structural analysis of AAV capsids in conjunction with mutagenesis studies has provided a better understanding of the molecular determinants of AAV capsid-glycan interactions. For instance, crystallographic and/or cryoelectron microscopy-based analysis have enabled mapping the role of critical amino acid residues involved in heparan sulfate recognition by AAV2, AAV-DJ, AAV3B and AAV6 capsids (17-19, 22, 24, 37). Recently the binding site of galactose on AAV9 capsids was mapped using a combination of mutagenesis and computational modeling tools (5). The structural/molecular basis for recognition of sialo/asialoglycans by the corresponding AAV serotypes is currently unknown.
Despite the accruing knowledge of AAV-glycan interactions in vitro, the underlying molecular basis for differential tissue tropisms displayed by AAV serotypes in vivo is not well understood. For instance, AAV2, which utilizes heparan sulfate proteoglycans for cell surface attachment is a hepatotropic strain, while AAV1, which binds N-linked α2,3-/α2,6-sialic acid displays cardiac/muscle tropism (4). In contrast, AAV9, which displays systemic, multi-organ tropism within different hosts (7, 15, 38) utilizes terminal galactose as its primary receptor (6, 28). The current study seeks to understand exactly how variations in host glycosylation patterns can affect AAV tissue tropism in mice, using AAV9-galactose interactions as a model. The study hinges on a two-pronged approach, the first of which exploits our preliminary observation that tissue sialo/asialoglycan levels can be readily modulated by enzymatic treatment in vivo. Briefly, intravenously administered sialidase afforded a dramatic increase in the density of asialoglycans (terminal galactose residues) across multiple tissues, which markedly altered the systemic behavior of AAV9 in mice. In the second part of this study, we carried out the systemic evaluation of a mutant AAV9 displaying low glycan binding avidity in normal mice. Our results demonstrate that altered tissue glycosylation patterns and/or glycan binding affinity can result in sharply contrasting liver uptake and blood circulation profiles of AAV in vivo.

RESULTS

Intravenous sialidase alters tissue surface glycosylation patterns in mice. Using lectin staining, we analyzed the endogenous constitution of sialylated glycans in major mouse organs as well as the effect of administering intravenous sialidase on tissue surface glycosylation patterns. First, BALB/c mice were intravenously injected with PBS or recombinant sialidase from V. cholerae 3 hours prior to tissue harvesting/fixation. Sections of major organs were then
stained with FITC-labeled lectins, specifically, ECL, which recognizes terminal galactose moieties on Gal(β1,4)N-GlcNAc or MALI, which binds both α2,3-sialylated and sulfated forms of Gal(β1,4)N-GlcNAc. As shown in Figure 1A, FITC-ECL staining is barely discernible, while substantial FITC-MALI staining is observed in murine heart, liver, brain and muscle. These observations suggest that the endogenous expression of asialoglycans containing terminal galactose is low when compared to that of α2,3-sialylated glycans in normal mouse tissues. In contrast, intravenous injection of recombinant sialidase markedly enhanced FITC-ECL staining intensity in heart, liver and muscle tissue, while MALI staining showed a modest decrease (Figure 1B). Interestingly, murine brain displayed ECL staining potentially localized to blood vessels alone, while MALI staining remained more or less unaffected upon sialidase treatment (Figure 1B). These results correlate well with earlier in vitro studies (28) and suggest that intravenous sialidase can effectively desialylate glycoproteins and glycolipids revealing the underlying β1,4-galactose moiety in major organs in vivo. We then exploited these observations to further evaluate the impact of increasing cognate glycan receptor density on AAV9 tropism in vivo.

High glycan binding avidity redirects systemic AAV9 to the liver. We analyzed the effect of enzymatic desialylation on the transduction profile of AAV9 vectors packaging a chicken β-actin (CBA) promoter-driven firefly luciferase transgene in vivo. Briefly, AAV9 vectors were injected into the tail vein at 3 hours following intravenous administration of recombinant sialidase. To track the kinetics of luciferase transgene expression, live animal bioluminescence images were taken at different time intervals post-injection (Figure 2A). As seen in Figure 2A, AAV9 displays a systemic transduction profile in the PBS treated mouse group (left panel), consistent with previous reports (11, 26, 38). In the sialidase treated group (Figure 2A, right panel), a predominantly liver-centric bioluminescent signal is observed, accompanied by decreased signal in other systemic regions. Luciferase transgene expression and viral genome copy

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number in different tissue lysates were quantified at 2 weeks and 3 days post-injection, respectively. As shown in Figure 2B, the transduction efficiency of AAV9 in the liver is ~5-fold higher upon sialidase treatment. Further, a substantial decrease in transduction efficiency within the heart (~10-fold) and skeletal muscle (~14-fold) is observed. Transgene expression levels in the murine brain are relatively low in comparison with other organs and show no significant change upon sialidase treatment. Consistent with these observations, the biodistribution of viral genomes in the liver is increased by ~7-fold in the liver (Figure 2C). Correspondingly, viral genome copy numbers are decreased in the brain, heart and skeletal muscle are decreased (ranging from 2 to 5-fold) upon sialidase treatment. These results suggest that surface glycan density in host tissues strongly influences AAV tissue tropism. In particular, increased availability of the galactose receptor in host tissue appears to redirect AAV9 capsids from major systemic organs towards the liver.

High glycan binding avidity results in AAV9 sequestration within liver endothelial and Kupffer cells. To understand the mechanism(s) underlying increased liver accumulation of AAV9, we analyzed liver sequestration of viral capsids through immunohistology. Briefly, mice were injected with AAV9 vectors 3 hours post-sialidase or PBS treatment. At 15 minutes post-vector administration, liver and heart were harvested and processed as described in materials and methods. Following PBS pre-treatment, we observed sporadic immunostaining for AAV9 capsids partially overlapping with endothelial cells defined by FcγRII/III/IV staining (anti-CD16/CD32) or other cell types within the liver (Figure 3A). In contrast, sialidase pre-treatment resulted in increased liver uptake of AAV9 capsids consistent with increased galactose density (Figure 3A). Further, increased immunofluorescence associated with AAV9 capsids almost completely co-localized with liver sinusoidal endothelial cells upon sialidase treatment (Figure 3A). We also observed substantially increased co-localization of AAV9 capsids with Kupffer cells (anti-F4/80) in addition to sinusoidal endothelial cells within the liver (Figure 3B). These
results confirm the earlier observation that high glycan density in host tissues redirects AAV9 from systemic organs to the liver. The underlying mechanism can be attributed to markedly increased sequestration of AAV9 capsids within the liver sinusoidal endothelium and Kupffer cells.

**Decreased glycan binding avidity detargets AAV9 from the liver.** We previously engineered a class of AAV9 mutants, which have selectively lost their ability to transduce the liver (26). As shown in the previous study, one such mutant, AAV9-W503R displays markedly lower transduction efficiency (> 1 log unit) in liver when compared to wild type AAV9. In the current study, we evaluated the ability of this mutant to bind surface-exposed galactose residues on Chinese hamster ovary (CHO) Lec2 cells, which are permissive to AAV9 infection *in vitro* (6, 28). As seen in Figure 4A, the AAV9-W503R mutant is deficient in cell surface binding, with a binding potential ($B_{\text{max}}/K_d$) that is ~4.5-fold lower than that observed for wild type AAV9. Correspondingly, this mutant displays transduction efficiency that is several orders of magnitude lower when compared to wild type AAV9 in CHO Lec2 cells at different multiplicities of infection (MOI) (Figure 4B). We then investigated the impact of such decreased glycan binding avidity on liver sequestration of AAV9 and the W503R mutant following intravenous administration. As seen in Figure 4C, immunohistological analysis reveals markedly lower immunostaining for AAV9-W503R in comparison with AAV9 capsids. Moreover, while AAV9 capsids co-localized with FcγRII/III/IV (anti-CD16/CD32 staining), which is specific for endothelial cells as well as liver parenchyma, the AAV9-W503R mutant weakly co-localized with endothelial cells alone (Figure 4C). In addition, consistent with earlier studies in our lab (26), we observed a lack of tdTomato reporter gene expression in hepatocytes for AAV9-W503R in contrast to wild type AAV9 (Figure 4D). Taken together, these results suggest that decreased glycan binding is the underlying basis for the liver-detargeted phenotype displayed by the AAV9-W503R mutant.
Vascular endothelium constitutes a limiting barrier for cardiac uptake of AAV9. We also evaluated the impact of altering glycan binding avidity on cardiac tropism of AAV9 in mice. As seen in Figure 5, immunostaining for AAV9 capsids (MAb ADK9, red) as well as the endothelial marker FcγRII/III/IV (anti-CD16/CD32 staining) is markedly lower in comparison to that observed in liver sections (Figure 3). Moreover, unlike liver, where viral particles are dispersed amongst parenchymal and non-parenchymal cell types, AAV9 capsids predominantly co-localize with the vascular endothelium in the murine heart. This observation supports the notion that transvascular transport is a likely prerequisite for viral entry into cardiomyocytes as proposed earlier (16). Interestingly, despite the dramatic increase in galactose receptor density as demonstrated by FITC-ECL staining throughout cardiac tissue following sialidase pre-treatment, no changes in the pattern or extent of cardiac uptake of AAV9 capsids were observed when compared to PBS-treated control. Instead, AAV9 capsids co-localized with cardiac endothelium in a manner similar to PBS-treated control animals. In addition, immunostaining for the low binding affinity mutant (AAV9-W503R) was significantly lower at this early time interval potentially due to decreased endothelial surface interactions.

Altered glycan binding avidity affects the blood circulation profile of AAV9. Several reports have demonstrated that AAV9 displays prolonged blood circulation in comparison with other AAV strains in mice (16, 38). We hypothesized that the latter phenomenon could arise from low endogenous levels of terminal galactose residues in murine tissue. To test this hypothesis, we first treated mice with intravenous sialidase prior to AAV9 administration in order to increase galactose receptor density in murine tissues as described earlier. Viral genome copies in blood were then collected at different time intervals, quantitated by Q-PCR analysis and the data fit to a bi-exponential, two compartment model (Table 1) to obtain different pharmacokinetic parameters (Tables 2 & 3). As seen in Figure 6A, high glycan binding avidity results in rapid clearance of AAV9 from the blood (solid circles), while the low binding avidity
mutant AAV9-W503R appears to persist longer in blood circulation in comparison to AAV9 circulating under normal conditions. Specifically, increasing tissue glycan density by sialidase treatment results in ~4 times faster elimination half-life ($t_{1/2,e}$). In contrast, the AAV9-W503R mutant, which is deficient in galactose binding, is eliminated twice as slowly (~10 hrs) from the blood circulation when compared to AAV9 alone (~4.7 hrs). Further, while the half-life of viral dissemination from blood to tissue ($t_{1/2,dis}$) is reduced by ~2-fold upon sialidase treatment, this parameter is extended from ~0.4 hrs to ~1 hr (2.5-fold increase) for the W503R mutant. Consistent with these observations, we note that the blood-to-organ distribution rate constant ($k_{12}$) and organ-to-blood distribution rate constant ($k_{21}$) are comparable in mice injected with AAV9 alone. In contrast, $k_{12}$ for AAV9 is ~7-fold higher than $k_{21}$ in mice pretreated with sialidase supporting rapid tissue dissemination of injected virus particles. Further, for the AAV9-W503R mutant, both the aforementioned rate parameters are lower in comparison with AAV9, consistent with prolonged blood residence time. Taken together, these results confirm that the capsid-glycan interactions profoundly affect the pharmacokinetics of AAV9.

**DISCUSSION**

The liver is a prominent systemic organ featuring hepatocytes as well as a large proportion of cells belonging to the reticuloendothelial system. Due to the large volume of circulating blood that filters through the liver, the sinusoidal endothelial and Kupffer cells in this organ play a major scavenging role in the uptake of various macromolecules and pathogens (27, 29). A particularly well-studied example in this regard is the adenoviral sequestration within the liver (8, 9, 13, 34). Most, if not all naturally occurring isolates of AAV studied to date, display a moderate-to-high transduction efficiency within the liver (38). However, whether liver
Sequestration mechanisms similar to those observed for adenoviruses prevail in the case of AAV is not known.

In the current study, we investigated the potential role of glycan receptor binding avidity on AAV liver sequestration and systemic spread using AAV9-galactose interactions as a model. Notably, the transduction profile and in vivo biodistribution of AAV9 were altered from systemic to hepatotropic under high glycan binding avidity conditions. These observations are corroborated by immunostaining studies, which revealed that increasing glycan receptor binding avidity potentiates AAV9 sequestration within the liver sinusoidal endothelium and Kupffer cells. In corollary, the AAV9-W503R mutant displays low galactose binding affinity and displayed decreased liver uptake in mice. Further, our results suggest that high affinity receptor sites for AAV9 in mice are constitutively low. This observation is not surprising, since it is well known that glycans containing β1,4-linked galactose are extended by sialylation or sulfation, although formation of poly-N-acetyllactosamine (Galβ1,4GlcNAcβ1-) units has also been demonstrated earlier (32). The resulting modest glycan binding avidity could explain the underlying basis for widespread, systemic tropism and prolonged blood circulation displayed by AAV9 in comparison to other AAV serotypes in vivo. Strikingly, high glycan binding avidity appears to adversely affect AAV9 uptake and transduction in peripheral tissues such as cardiac and skeletal muscle. This effect can likely be attributed to the rapid blood clearance of AAV9 under such conditions and highlights the limiting role of vascular endothelium in viral dissemination. Our conclusions are further supported by earlier studies by Nakai and others (16), which demonstrate that transendothelial transport of AAV9 is a capacity-limited process that constitutes a significant barrier towards viral uptake by cardiomyocytes, but not hepatocytes.

Why is the AAV9-W503R mutant selectively detargeted from the liver, while transduction in peripheral tissues such as the heart and skeletal muscle remain unaffected (26)? In the current study, we determined that this mutant (as well as other liver-detargeted AAV9 mutants,
Shen and Asokan, unpublished) displays low galactose binding avidity in vitro and reduced endothelial binding in vivo. These results are consistent with W503R being recently identified as one of the key residues involved in galactose recognition by AAV9 capsids (5). A potential explanation for this phenomenon is that low glycan binding avidity results in the doubling of distribution half-life ($t_{1/2,\text{dis}} \sim 1$ hr) as well as elimination half-life ($t_{1/2,\text{e}} \sim 9$ hrs) of the mutant when compared to wild type AAV9 (Table 3). Thus, it is likely that the AAV9-W503R mutant significantly benefits from repeated circulation through cardiac tissue, where traversing the vascular endothelium appears to be a capacity-limited process. Such transendothelial transport of viral particles might be redundant for transduction in liver, which contains open fenestrae that allow free exchange of material between blood and the space of Disse (29). These results are further corroborated by earlier studies with AAV serotype 2, wherein, abrogation of capsid binding to heparan sulfate yields liver-detargeted AAV2 mutants (14). One such mutant, AAV2i8, which persists longer in blood than other AAV strains, displays robust cardiac and skeletal muscle tropism with ability to traverse the vascular endothelium (3). Based on a collective interpretation of results, we propose mathematical models that will help predict the impact of capsid-glycan interactions on AAV blood clearance as well as transport between the central (blood) compartment and major peripheral (liver) compartment (Figures 6B and 6C).

High glycan receptor binding avidity will likely increase the rate of viral dissemination from blood to liver ($k_{12}$) as well as rapid viral elimination from blood ($k_e$) by excretory mechanisms (Figure 6B). In contrast, when glycan binding avidity is low, both dissemination and elimination rate constants are decreased resulting in significantly longer blood circulation times (Figure 6C). It should be noted that while glycan binding avidity appears to influence systemic fate, the secondary receptor(s) for AAV9 likely contribute significantly towards transduction in vivo. Therefore, although virus-glycan interactions alone are unlikely to provide a complete understanding of the biology of AAV, it is tempting to speculate that the latter models could...
potentially help predict liver sequestration of AAV in different species on the basis of their respective glycosylation patterns.

In summary, our studies offer new insight into the role of glycans in determining AAV tropism \textit{in vivo}. In addition to resolving the mechanistic basis of liver-detargeted AAV vectors, we provide a potential general strategy for engineering any AAV strain by modulating capsid-glycan interactions. Further, the results described herein underscore the importance of understanding species-specific variation in glycosylation patterns. For instance, striking differences in sialylation patterns between different tissues in mice, rats, chimpanzees and humans have been reported (2). Such information could guide rational selection of preclinical animal models for evaluating AAV vectors and might help better predict clinical gene therapy outcomes.

**MATERIALS AND METHODS**

**Biochemical reagents.** Recombinant sialidase (neuraminidase type III from \textit{V. cholerae}) was obtained from Sigma-Aldrich (St. Louis, MO). \textit{Erythrina Cristagalli} (ECL) and \textit{Maackia Amurensis I} (MAL-I) lectins were purchased from Vector Labs (Burlingame, CA). Monoclonal antibodies (MAb) against endothelium-specific marker FcγRII/III/IV (rat 2.4G2 anti-CD16/CD32) (9) was obtained from BD Biosciences (San Jose, CA), and Kupffer cell-specific marker (rat anti-F4/80) (9) was obtained from Abcam (Cambridge, MA). Fluorophore-conjugated secondary antibodies (goat anti-rat or anti-mouse) were obtained from Invitrogen (Carlsbad, CA). The ADK9 mouse anti-capsid MAb recognizing intact AAV9 capsids was a kind gift from Dr. Jürgen Kleinschmidt (German Cancer Research Centre, DKFZ, Heidelberg, Germany).
AAV production. HEK293 cells were maintained at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin-streptomycin-amphotericin B. The following plasmids were generated in the lab as described earlier (26) or obtained from the UNC vector core facility: (1) the pXR9 plasmid encoding rep2 and cap9; (2) the plasmid pXX6-80 containing adenoviral helper genes; and (3) the vector cassettes, pTR-CBA-Luc or pTR-CBA-tdTomato, encoding the Chicken beta-actin (CBA) promoter and luciferase or tdTomato reporter transgene, respectively. AAV9 vectors packaging TR-CBA-Luc or TR-CBA-tdTomato were produced by the triple transfection method (10) followed by cesium chloride density gradient ultracentrifugation and dialysis against 1x phosphate buffered saline (PBS) overnight with two changes. Viral titers were determined by quantitative PCR using primers (IDT Technologies, Ames, IA) specific for the CBA promoter (forward 5′-CGT CAA TGG GTG GAG TAT TT-3′; reverse 5′-GCG ATG ACT AAT ACG TAG ATG-3′) or Luc transgene region (forward 5′-AAA AGC ACT CTG ATT GAC AAA TAC -3′; reverse 5′-CCT TCG CTT CAA AAA ATG GAA C-3′). Viral titers for different vector preps typically ranged from 2-5 x 10^{12} vector genome-containing particles per mL (vg/mL).

Cell surface binding assay. CHO Lec2 cells deficient in terminal sialic acid moieties were seeded at a density of 10^4 cells/well in 96-well plates and maintained in an incubator at 37 °C and 5% CO2 overnight. Prior to adding virus particles, cells were pre-chilled to 4 °C for 30 min, followed by incubation with AAV9 or AAV9-W503R particles at MOI of 1x10^2, 5x10^2, 1x10^3, 5x10^3, 5x10^4, 5x10^4, 5x10^5, 5x10^5 vg/cell for 1.5 hrs at 4 °C. Cells were then subjected to three washes with ice-cold 1x PBS to remove unbound virions. Cell surface-bound virions were collected along with cell lysates following three freeze-thaw cycles and vg copy numbers/cell determined using quantitative PCR as outlined earlier. Binding curves were generated using
GraphPad Prism 5 software by applying the single site binding model ($Y = \frac{B_{\text{max}} \cdot X}{(K_d' + X)}$), where $Y$ represents the number of bound virions/cell determined by quantitative PCR; $X$ represents MOI; $B_{\text{max}}$ is the maximum binding capacity, and $K_d'$, the observed disassociation constant. Binding potential is defined as $\frac{B_{\text{max}}}{K_d'}$ and serves as a measure of glycan binding avidity.

**Live animal studies.** All *in vivo* experiments were carried out using 6–8-week old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) maintained and treated in accordance with National Institutes of Health guidelines and as approved by IACUC at UNC-Chapel Hill. Sialidase (neuraminidase type III from *V. cholerae*, 200 milliunits) or PBS was administrated through intravenous injection 3 hrs prior to AAV9-CBA-Luc (5x10$^{10}$ vg) through the same route. Luciferase transgene expression in live animals was monitored at indicated time points for each experiment using a Xenogen IVIS Lumina® imaging system (Perkin Elmer/Caliper Life Sciences, Waltham, MA) following intraperitoneal injection of D-luciferin substrate (120 mg/kg; Nanolight, Pinetop, AZ). Bioluminescent image analysis was conducted using the Living Image software® (Perkin Elmer/Caliper Life Sciences, Waltham, MA) and luciferase expression reported in relative light units (photons/s per cm$^2$ per steradian).

**Quantitation of luciferase expression.** The same group of mice was sacrificed at indicated time points and the heart and liver harvested. Approximately 50 mg of each tissue was homogenized in 150 μl of passive lysis buffer (Promega, Madison, WI) using a Tissue Lyser II system (Qiagen, Valencia, CA). Tissue lysates were centrifuged at 8,000 rpm for 2 min to pellet debris and 50 μl of the supernatant transferred to 96-well plates for luminometric analysis using a Victor2® luminometer (Perkin Elmer, Waltham, MA). Total protein concentration in tissue lysates was determined using the Bradford assay (BioRad, Hercules, CA).
Quantitation of viral genome biodistribution. Approximately 100 μl of supernatant from tissue lysates obtained as mentioned above was processed using a DNeasy kit (Qiagen, Valencia, CA) to extract host and vector genomic DNA. Vector genome (Luc) and mouse lamin gene (housekeeping gene serving as internal standard) copy numbers were determined from 100 ng of total extracted DNA using quantitative PCR as described earlier.

Immunohistology. For lectin staining studies, sialidase (neuraminidase type III from V. cholerae, 400 milliunits) in 150 μl of PBS or PBS alone was first administrated via tail vein injection. At 3 hours post-administration, mice were anesthetized with intraperitoneal injection of avertin (0.2 ml/10 g of a 1.25% solution) and perfused transcardially with 20 ml of PBS, then 20 ml of freshly prepared 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS. Cardiac, liver, brain, and muscular tissues were fixed at 4 °C overnight, and then sectioned into 40 μm slices using a Leica vibrating blade microtome (Leica Microsystems, Buffalo Grove, IL). The sections were blocked in 10% goat serum in 1xPBS containing 0.1% Triton X-100 (PBS-T) for 1 hr at room temperature prior to incubation with primary antibodies (10 μg/ml CD16/CD32 or F4/80 MAb and 1:10 dilution of ADK9 in hybridoma media) for 24 hrs at 4 °C. Antibody binding was localized using fluorophore-conjugated secondary antibodies diluted 1:1000 in 3% goat serum in PBS-T for 1 hr at 4 °C. Specifically, Alexa Fluor 647 goat anti-rat IgG (H+L) (Invitrogen A21247, 2mg/ml, 1:500 dilution) was used to recognize rat anti-mouse-CD16/CD32 and rat anti-mouse-F4/80, and Alexa Fluor 594 goat anti-mouse IgG (H+L) (Invitrogen A11005, 2mg/ml, 1:500 dilution) was utilized to detect ADK9 MAb, which binds to intact AAV9 capsids. When indicated, the sections were further stained in 30 μg/ml of FITC-labeled ECL or MAL-I lectins in 3% blocking solution. After three washes with PBS to get rid of unbound antibodies and lectins, the sections were mounted under cover slips in Prolong® gold anti-fade reagent with DAPI (Invitrogen P36935). Tissues harvested from untreated mice were utilized as controls. Four
color fluorescence images were acquired using a Zeiss 710 confocal laser scanning microscope equipped with a spectral detection system for finer separation of fluorochromes. Image processing was carried out using LSM viewer and Image J® softwares.

To compare the transduction profile of AAV9 and AAV9-W503R in live animals, PBS (mock treatment), AAV9-CBA-tdTomato (5x10^{10} vg), and AAV9-CBA-tdTomato (5x10^{10} vg) were injected to BALB/c mice through tail vein. 2 weeks post infection, animals were anesthetized using intaperitoneal avertin (0.2 ml/10g of a 1.25% working solution), followed by transcardial perfusion with 20 ml of PBS and 20 ml of 4% paraformaldehyde in PBS. Tissues were fixed in 4% paraformaldehyde in PBS for overnight at 4˚C, and sectioned into 40 μm slices using a Leica vibrating blade microtome. Tissue sections were mounted under cover slips using Prolong® gold anti-fade reagent with DAPI. Red fluorescent images were taken using an Olympus epifluorescence microscope equipped with a 10x objective and a Hamamatsu digital camera.

**Pharmacokinetic analysis.** Sialidase (neuraminidase type III from *V. cholera*, 200 milliunits) or 100 μl of PBS was injected through intravenous injections via the mouse tail vein. AAV9-CBA-Luc (5x10^{10} vg) was injected through the same route 2 hrs later. For pharmacokinetic analysis, 10 μl of blood was drawn from the tail vein through nicking at the different time intervals into heparinized glass capillary tubes (Fisherbrand Hematocrit®). Viral DNA was extracted from the blood samples using a DNeasy kit (Qiagen, Valencia, CA) and further quantified by qPCR as described earlier. Blood clearance of viral genomes was plotted as a function of viral genome copy number vs. time and the data fit into a bi-exponential model using least-square regression analysis in GraphPad Prism® software. Distribution and elimination half-life as well as different rate constants based on a two compartment pharmacokinetic model were calculated using...
equations outlined in Table 1 as described earlier (25). Pharmacokinetics parameters, standard errors, and goodness of fit from curve-fitting are listed in Tables 2 and 3.

Statistics. All data are expressed as mean ± SEM unless indicated otherwise. The two-tailed unpaired t test was utilized for all statistical analysis and P values < 0.05 were considered significant unless indicated otherwise. Statistical evaluation of pharmacokinetic parameters and goodness of fit are provided in Supplementary Table S2.

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REFERENCES


FIGURE LEGENDS

Figure 1. Intravenous sialidase alters endogenous tissue glycosylation patterns in vivo.

(A) Fluorescent lectin staining of heart, liver, brain and skeletal muscle tissue harvested from BALB/c mice. FITC-ECL was used to detect tissue glycans containing terminal β1,4-Galactose and FITC-MALI detects α2,3-sialylated glycans. Confocal micrographs were obtained using a Zeiss 710 Confocal Laser Scanning Microscope with a 40x objective at zoom 0.6. Scale bar = 50 μm. (B) Fluorescent lectin staining of heart, liver, brain and skeletal muscle harvested from BALB/c mice administered intravenous sialidase to enzymatically remove α2,3-sialic acid residues in vivo.

Figure 2. High glycan binding avidity redirects systemic AAV9 to the liver.

(A) Live animal bioluminescence images of AAV9-mediated luciferase transgene expression in BALB/c mice. Animals were injected with PBS (control, left panel) or recombinant sialidase (right panel) 3 hours prior to AAV9-CBA-Luciferase administration via tail vein. Images were obtained using a Xenogen IVIS Lumina system at 2 days, 4 days, and 7 days post-administration (n=3). Rainbow scale represents relative light units (RLU) expressed in photons per sec per cm² per steradian. (B) Luciferase transgene expression in brain, heart, liver, skeletal muscle tissue lysates. Mice were intravenously injected with PBS (white bars) or sialidase (grey bars) 3 hours prior to AAV9. Tissues were harvested at 2 weeks post-administration. Luciferase transgene expression (RLU) is normalized to total cellular protein in tissue lysate (n=3). Data are represented as mean ± SEM. (C) AAV9 vector genome biodistribution in brain, heart, liver and skeletal muscle tissues harvested from BALB/c mice pre-treated with PBS (white bars) or sialidase (grey bars) at 3 days post-administration (n=3). Viral genome copy numbers in different tissues/animals were obtained using quantitative PCR and normalized to total copy number of the constitutive
mLamin gene in host genomic DNA. Primer sequences utilized for QPCR are listed under experimental procedures. Data are represented as mean ± SEM.

**Figure 3. High glycan avidity potentiates AAV9 sequestration by liver sinusoidal endothelium and Kupffer cells.** (A) Liver sections were obtained 15min post-injection of AAV9 in BALB/c mice pre-treated with intravenous PBS (middle row) or sialidase (bottom row). Tissue sections were stained with fluorescent lectin to detect β1,4-galactose, FITC-ECL (green); anti-AAV9 capsid antibody, ADK9 (red) and anti-CD16/CD32 endothelial cell marker antibody (magenta). Color images were merged along with nuclear DAPI staining (blue) to generate the overlay panel. Untreated mouse liver is shown as control (top row). Large white arrows indicate liver sinusoidal endothelial cells and small white arrows indicate non-endothelial cells. Fluorescent micrographs were obtained using a Zeiss 710 Confocal Laser Scanning Microscope with a 63x objective. Scale bar = 50 μm. (B) Liver sections obtained from BALB/c mice pre-treated with intravenous PBS or sialidase and immunostained with FITC-ECL (green), ADK9 (red), anti-F4/80 Kupffer cell marker antibody (magenta) and nuclear DAPI staining (blue). Large white arrows indicate liver sinusoidal endothelial cells and small white arrows indicate Kupffer cells.

**Figure 4. Low glycan binding avidity detargets AAV9-W503R from the liver.** (A) The AAV9-W503R mutant displays decreased binding affinity to terminal galactose residues on the surface of CHO Lec2 cells lacking sialic acid (n=4). Curve fitting based on a single-binding site model was utilized to obtain binding potential values (B_{max}/K_{d}') for the mutant and parental AAV9. Data are represented as mean ± SEM. (B) Transduction efficiency of AAV9 and AAV9-W503R on CHO Pro5 (constitutive glycan composition) and Lec2 cells (sialic acid-deficient) at different multiplicities of infection (n=4). Luciferase transgene expression in cell lysates was analyzed 24 hrs post-infection **p < 0.01; ***p < 0.005. Data are represented as mean ± SEM. (C) AAV9 and
AAV9-W503R accumulation in the liver of BALB/c mice at 15 min post-intravenous administration. Immunostaining was carried out using anti-AAV9 capsid antibody, ADK9 (red); anti-CD16/CD32 endothelial cell marker (magenta) and nuclear DAPI staining (blue). Fluorescent images were collected as described earlier. Large white arrows indicate liver sinusoidal endothelial cells and small white arrows indicate non-endothelial cells. Scale bar = 20 μm. (D) Transgene expression (tdTomato reporter) mediated by AAV9 and AAV9-W503R in liver and heart in BALB/c mice (n=3). Tissues were harvested and imaged 2 weeks post-infection with a 10x objective. BALB/c

Figure 5. Vascular endothelial cells limit cardiac uptake of AAV9 capsids. Confocal micrographs of cardiac tissue sections harvested from BALB/c pre-treated with PBS (second row) or sialidase (third row) obtained at 15min post-intravenous AAV9 injection. Immunostaining was carried out using FITC-ECL (green), ADK9 (red), anti-CD16/CD32 (magenta) and nuclear DAPI staining (blue). Co-localization of mutant AAV9-W503R with vascular endothelium in the heart (bottom row) and cardiac tissue sections from untreated BALB/c mice (top row) are also shown. Large white arrows indicate cardiac endothelium. Scale bar = 50 μm.

Figure 6. Glycan binding avidity affects the blood circulation profile of AAV9. (A) Blood circulation kinetics of the AAV9-W503R mutant (●) or AAV9 in BALB/c mice pre-treated with PBS (○) or sialidase (▲). Viral genome copy numbers in blood obtained at 15min, 30min, 1 hr, 3 hrs, 6 hrs and 24 hrs post-administration were obtained by quantitative PCR (n=3). Curve fitting based on a bi-exponential, two-compartment model was utilized to obtain pharmacokinetic parameters listed in Table 1. Data are represented as mean ± SEM. Equations, goodness of fit and statistical analysis are listed in Supplementary Tables S1 and S2. (B) Proposed mathematical model for predicting the impact of high glycan binding avidity on viral blood clearance and liver sequestration. Rates of viral elimination from blood (k_e), viral dissemination
from blood-to-liver ($k_{12}$) and liver-to-blood ($k_{21}$) are shown. Bold arrows indicate faster rates of
dissemination and clearance from blood. This model specifically explains the pharmacokinetics
of wild type AAV9 following enzymatic desialylation in mice. (C) Proposed mathematical model
for predicting the impact of low glycan binding avidity on viral blood clearance and liver
sequestration. Different rates ($k$ values) are shown as above. Dotted arrows indicate slower
rates of dissemination and clearance from blood. This model specifically explains the
pharmacokinetics of mutant AAV9-W503R in mice.
<table>
<thead>
<tr>
<th>Table 1. Pharmacokinetic equations for a two compartment model</th>
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<tbody>
<tr>
<td>Bi-exponential equation describing AAV9 pharmacokinetics:</td>
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<tr>
<td>[ C_p = A \times e^{-\alpha x} + B \times e^{-\beta x} ]</td>
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<tr>
<td>Tissue-to-blood compartment distribution rate constant:</td>
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<tr>
<td>[ k_{21} = \frac{A \cdot \beta + B \cdot \alpha}{A + B} ]</td>
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<tr>
<td>Blood elimination rate constant:</td>
</tr>
<tr>
<td>[ k_e = \frac{\alpha \cdot \beta}{k_{21}} ]</td>
</tr>
<tr>
<td>Blood-to-tissue compartment distribution rate constant:</td>
</tr>
<tr>
<td>[ k_{12} = \alpha + \beta - k_{21} - k_e ]</td>
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<tr>
<td>Distribution half-life:</td>
</tr>
<tr>
<td>[ t_{1/2}^{\text{distribution}} = \frac{\ln 2}{k_{12}} ]</td>
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<tr>
<td>Elimination half-life:</td>
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<tr>
<td>[ t_{1/2}^{\text{elimination}} = \frac{\ln 2}{k_e} ]</td>
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</tbody>
</table>

# Cp is the viral genome copy number in plasma, x is the time interval
Table 2. Pharmacokinetic parameters, standard errors, and goodness of fit from curve-fitting blood circulation data to a bi-exponential, two-compartment model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PBS</th>
<th>Sialidase</th>
<th>PBS</th>
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<tbody>
<tr>
<td>Virus</td>
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<td>AAV9</td>
<td>AAV9</td>
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<tr>
<td>$Y_0$</td>
<td>$6.86 \times 10^{10} \pm 9.81 \times 10^9$</td>
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<tr>
<td>$A$</td>
<td>$3.75 \times 10^{12} \pm 5.55 \times 10^{10}$</td>
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<tr>
<td>$B$</td>
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<td>$1.10 \times 10^{12} \pm 2.49 \times 10^{10}$</td>
<td>$4.68 \times 10^{12} \pm 8.83 \times 10^{11}$</td>
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<tr>
<td>$\alpha$</td>
<td>$3.509 \pm 1.12$</td>
<td>$4.602 \pm 0.7836$</td>
<td>$1.752 \pm 3.566$</td>
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<td>$\beta$</td>
<td>$6.826 \times 10^{-2} \pm 4.874 \times 10^{-3}$</td>
<td>$7.055 \times 10^{-2} \pm 7.696 \times 10^{-3}$</td>
<td>$3.884 \times 10^{-2} \pm 6.423 \times 10^{-2}$</td>
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<tr>
<td>$R^2$</td>
<td>0.9995</td>
<td>0.9999</td>
<td>0.7250</td>
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Table 3. Parameters describing the pharmacokinetics of AAV9 under different glycan binding avidity conditions *in vivo*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AAV9</th>
<th>AAV9+Sialidase</th>
<th>AAV9-503R</th>
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<tbody>
<tr>
<td>Glycan binding avidity/Parameters</td>
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<td>+++</td>
<td>+</td>
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<tr>
<td>$k_{21}$ (h$^{-1}$)</td>
<td>1.63</td>
<td>0.57</td>
<td>0.97</td>
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<tr>
<td>$k_e$ (h$^{-1}$)</td>
<td>0.15</td>
<td>0.57</td>
<td>0.07</td>
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<tr>
<td>$k_{12}$ (h$^{-1}$)</td>
<td>1.80</td>
<td>3.53</td>
<td>0.75</td>
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<tr>
<td>$t_{1/2,dis}$ (h)</td>
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<td>0.20</td>
<td>0.92</td>
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<tr>
<td>$t_{1/2,e}$ (h)</td>
<td>4.72</td>
<td>1.22</td>
<td>9.87</td>
</tr>
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