Superior Retinal Gene Transfer and Biodistribution Profile of Subretinal Versus Intravitreal Delivery of AAV8 in Nonhuman Primates

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PURPOSE. To investigate shedding and biodistribution characteristics of recombinant adeno-associated virus serotype 8 (rAAV8) after single-dose subretinal or intravitreal injection in nonhuman primates (NHP, Macaca fascicularis) as a surrogate for environmental hazard and patient safety.

METHODS. In a study for regulatory submission, 22 NHP were divided into four cohorts receiving either single subretinal injections of vehicle or clinical grade rAAV8 (1 \times 10^{11} or 1 \times 10^{12} vector genomes (vg)) versus single intravitreal application of 1 \times 10^{12} vg. Viral shedding and biodistribution were monitored in biofluids for up to 91 days, followed by necropsy and tissue harvesting of all major organs, the visual pathway, and lymphatic tissue. Quantification of vector genomes was done by quantitative (q)PCR.

RESULTS. Shedding occurred in a dose-dependent manner in all biofluids and persisted for a maximum of 7 days. Intravitreal delivery led to increased and persistent (up to 13 weeks) distribution of vector genomes in blood and draining lymphatic tissue, increased off-target deposition, and inefficient gene transfer to the retina. No vector targeting of the germ line was observed in any cohort.

CONCLUSIONS. These data illustrate that subretinal application of rAAV8 leads to a more favorable biodistribution profile compared to intravitreal injections. Extraocular biodistribution is limited after subretinal delivery, while intravitreal injection leads to both greater and more persistent systemic exposure, evident in blood and lymphatic tissues. With the knowledge on the dynamics of shedding in a setting mimicking clinical application, guidelines can be developed to refine clinical trial protocols to reduce the risk for trial subjects and their environment.

Keywords: AAV8, nonhuman primates, biodistribution, shedding, subretinal versus intravitreal...
surgical procedures used in patients. These data are expected to be relevant to all ocular gene therapies utilizing the adeno-associated virus serotype 8 (AAV8) capsid and possibly other serotypes with similar tropism, as the affinity of capsid surface epitopes toward local receptors helps to define their distribution pattern. Recent evidence of innate and adaptive immunity toward AAV epitopes increases the significance of these findings.10,11

**Materials and Methods**

Cynomolgus monkeys (nonhuman primates, NHP) were treated and cared for at the Covance Preclinical Services GmbH test facility in Muenster, Germany. The study was conducted with great care to ensure the well-being of the animals and was approved by the local authorities (Regierungsspraesidium of North Rhine Westphalia). All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and in full compliance with the guidelines of the European Community (EUVD 86/609/EEC) for the care and use of laboratory animals, as well as in accordance with Good Laboratory Practice (GLP) standards as defined by German GLP monitoring authorities and in compliance with U.S. Food and Drug Administration and Good Laboratory Practice regulations.

**Animals**

A total of 22 NHP were assigned to four study cohorts. Six animals (three of each sex) were assigned to cohorts 1 to 3 and treated with subretinal injections (SR). Cohort 1 received vehicle only: balanced salt solution, BSS (Alcon Laboratories [UK] Ltd., Camberley, UK) with 0.001% PF-68 surfactant (BASF, Ludwigshafen, Germany). Cohorts 2 and 3 received single SR of 1 × 10^11 vector genomes (vg) (low dose) or 1 × 10^12 vg (high dose), respectively. Cohort 4 consisted of four animals (two of each sex) and was given 1 × 10^12 vg (high dose) to mimic leakage from the SR setting. A temporal canthotomy was performed for improved access, and three transconjunctival sclerotomies were made after transillumination confirmed the location of pars plana. Vitrectomy was performed as completely as possible without affecting the lens. A localized retinal detachment was induced through SR of 50 μL BSS (Alcon Laboratories) using a 41-gauge cannula (DORC 1270.EXT; Dutch Ophthalmic Research Center [International], B.V., Zuidland, The Netherlands). Virus solution (200 μL) was injected into the preformed bleb using a foot pedal-controlled injection system (PentaSys II; Ruck GmbH, Eschweiler, North Rhine-Westphalia, Germany). Before recovery, subconjunctival cefuroxime (125 mg; Ratiopharm GmbH, Ulm, Baden-Württemberg, Germany) and dexamethasone (2 mg; Ratiopharm GmbH) were administered to the operated eye. Postoperative prophylactic treatment consisted of antibiotic (0.5% moxiﬂoxacin; Ratiopharm GmbH), and anti-phlogistic (1% prednisolone; Ratiopharm GmbH) eye drops given 5/day each in the treated eye for 2 weeks and prednisone (Ratiopharm GmbH) 1 mg/kg intramuscularly from day −2 until day 5.

**Biofluid and Tissue Sampling**

Bioﬂuid samples were harvested from all animals before dosing and on days 2, 3, 5, 7, and 31 for quantitative (q)PCR analysis. Blood was collected before dosing and 24, 48, 72, and 168 hours and at weeks 4 and 13 post dosing. Predose samples and samples collected on days 2 and 3 were analyzed in the first instance. The remaining samples were analyzed until there were two consecutive time points that were negative for vector genomes. Sample volumes used for analyses were 220 μL for blood, tear film, and nasal secretions, 100 μL for urine, and 50 μL for aqueous humor. For large organs, tissue samples were harvested at necropsy (91 days post dosing) with sterile, DNase-free single-use instruments and stored at <−70°C until DNA extraction. Where reported, whole organ weights, as well as individual sample weights, were documented. This enabled extrapolation of copy numbers per mg sample to copy numbers per (whole) organ.

**DNA Extraction and qPCR**

DNA was extracted from all tissue and blood samples prior to qPCR using QIAsymphony DSP DNA Mini kit with Qiagen Reagent DX, and the QIAsymphony DSP DNA Mini kit, respectively (Qiagen, Hilden, North Rhine-Westphalia, Germany). DNA concentrations were measured using spectrophotometry. Eight cryosections (20 μm thick) from whole eye cups were processed for each animal and pooled into one sample for extraction. Also, for heart, lung, and liver, three samples were extracted separately and the eluates pooled to result in one sample analyzed by qPCR. For all tissue samples and blood, 1 μg DNA was used per qPCR reaction. QIAsymphony DSP Virus/Pathogen kit for biofluids was used for the remaining samples. DNA concentration of these samples was not determined, due to the inclusion of carrier RNA in the extraction procedure. Here, samples of 5 μL were analyzed neat by qPCR. For presentation, results are normalized to 1 mL source material to improve interpretability. To validate the qPCR assay, a positive control (transgene plasmid) was serially diluted from 5 × 10^7 to 50 copies/reaction. The data generated from this dilution series were used to construct a standard curve for quantitative data analysis. Samples that tested positive after 40 cycles of amplification, but below 50 copies, were supposed to contain a nonquantifiable number of genomes per reaction (between 1 and 50), and thus deemed “<50 LLOQ.” Samples without amplification after 40 cycles were deemed “negative.” This validated qPCR assay was used to analyze each sample in...
TABLE 1. Surrogates Relevant to Clinical Practice

<table>
<thead>
<tr>
<th>Clinical Surrogates, Shedding</th>
<th>Maximal Shedding</th>
<th>Thresholds, Achieved in Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Cohort</td>
<td>On Day</td>
</tr>
<tr>
<td>Tear film</td>
<td>Low-dose SR</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>High-dose SR</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>High-dose IRT</td>
<td>3</td>
</tr>
<tr>
<td>Nasal secretions</td>
<td>Low-dose SR</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>High-dose SR</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>High-dose IVT</td>
<td>2</td>
</tr>
<tr>
<td>Urine</td>
<td>Low-dose SR</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>High-dose SR</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>High-dose IVT</td>
<td>2</td>
</tr>
<tr>
<td>Blood</td>
<td>Low-dose SR</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>High-dose SR</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>High-dose IVT</td>
<td>1</td>
</tr>
</tbody>
</table>

Tears and urine featured a response to both dose and route of administration. While dose escalation led to increased copy numbers and shedding duration, route of administration mostly affected copy numbers, rather than shedding duration, which was generally similar between subretinal and intravitreal injections. Blood samples did not show any vector presence after low-dose subretinal injections, and limited vector load after high-dose subretinal injections, while intravitreal administration led to immediate, strong, and persistent presence of vector in blood. First column: Groups. Second column: Time point of maximal shedding, vector genomes/mL of raw sample (cohort mean). Third column: Persistence of vector shedding, characterized by two thresholds: residual shedding = all samples testing at least below LLOQ, no shedding (none) = all samples negative.

RESULTS

Animal Dosing

All randomly assigned animals successfully underwent dosing per protocol within 3 consecutive days. Complete subretinal placement was evident in direct visualization through the operating microscope and involved the targeted macular area in all animals. All biofluid and tissue sampling could be performed according to standard operating procedures and in strict accordance with GLP guidelines.

Animals Injected With Vehicle Only

All tissue and biofluid samples taken from animals in the control cohort injected with vehicle tested negative for presence of transgenic DNA, with exception of one tear sample and two nasal swabs taken from one female animal. In these samples, transgenic DNA quantities detected were all below the lower level of quantification (LLOQ = 50 copies per reaction). All consecutive tear samples and nasal swabs from this animal tested negative, as did all other biofluid and/or tissues samples at all time points.

Animals Injected With Vector Solution

Shedding occurred to some degree in all animals injected with vector solution and in all types of biofluids tested. An overview of maximal amount of detected vector genomes/mL biofluid and duration of shedding by route of administration and dose is given in Table 1. Of the biofluids that shed into the environment (tears, nasal secretions, and urine) no sample tested above the LLOQ later than day 7. Maximal shedding in most animals was observed 2 days after injection. After SR, shedding was most pronounced in tear fluid, followed by nasal secretions and urine. After IVT, the largest amount of vector was also shed via tears, but was followed by urine and lastly nasal secretions. Table 1 presents time points when shedding of all samples of a cohort were below LLOQ (residual shedding), or below level of detection (no shedding). Figure 1 summarizes the qualitative changes of shedding status (positive, residual, or negative) per cohort and biofluid over the observed time frame. To characterize time dependency it introduces \(T_{1/2}\) as a semiquantitative “shedding status half-life”: the point in time (in days) after administration on which \(\geq50\%\) of a cohort’s samples test below LLOQ. Individual shedding results of all animals are shown in Figures 2 to 4, plotted in log scale against time of harvest, capturing both the variability between animals of the same cohort and differences between cohorts.

Individual Lacrimal Shedding Results

Shedding via tear film was subject to a pronounced dose effect (Figs. 1, 2; Table 1). In animals from the low-dose group, fewer copies were found in positive samples, and tears were negative after a shorter time, compared to the high-dose group. Subretinal application of the high dose led to a 33-fold increase in maximal shedding (cohort mean) compared to the low dose, and shedding persisted at quantifiable levels for at least 1 week after surgery. Route of administration also had distinct effects on shedding via tears. While fewer copies were detected following intravitreal compared to SR (with the same amount of vector applied), both groups featured quantifiable samples after 1 week. Where only four out of six animals from the low-dose group shed vector via tears at any given time point, all (6/6) animals from the high-dose subretinal and (4/4) intravitreal cohorts shed vector via tears over the course of the study. Individual results are displayed in Figure 2 and copy numbers/mL are given in Supplementary Material S1.

Individual Shedding Results via Nasal Secretions

Shedding via nasal secretions was less dependent on dosage than shedding through tears and urine (Figs. 1, 3; Table 1). Shedding in the low-dose subretinal cohort receded more quickly \((T_{1/2} \text{ after } 4 \text{ vs. } 7 \text{ days for high dose})\) and was slightly less prevalent \((5/6 \text{ animals})\), compared to the high-dose cohort \((6/6)\). In line with the slightly increased prevalence after high-dose injection, dose escalation by one log unit also entailed a modest 1.4-fold increase in maximal copy numbers (cohort...
Shedding prevalence per cohort and biofluid during the observed time frame. Switching between columns compares results of the same sample type (i.e., shedding via tear) over different cohorts (i.e., subretinal low versus high dose), while switching between rows compares different sample types in the same cohort. The most notable difference is visible in the bottom row of the graph, displaying vector presence in blood across the three cohorts. y-axes: sample status in % of animals in the given cohort, color-coded. x-axes: days after injection. $T_{1/2}$: $\geq 50\%$ of the cohort’s samples test below LLOQ or negative. Individual sample status is color-coded.
Like dose escalation, route of delivery had only a minor effect on shedding via nasal secretions. Between subretinal and intravitreal cohorts of the same dose, there was an equal prevalence (all animals in both groups) and duration of shedding, with 50% of animals in both cohorts featuring quantifiable qPCR results 7 days after surgery. A slight difference between the high-dose groups was found in a 1.7-fold elevation of copy numbers after SR, relative to intravitreal administration. Individual results are displayed in Figure 3 and copy numbers/mL are given in Supplementary Material S1.

Individual Shedding Results via Urine

Shedding via urine was strongly dose dependent (Figs. 1, 4; Table 1). Foremost, it was barely detectable and nonpersistent in the low-dose cohort. Due to the low-dose cohort’s low baseline, dose escalation by one log unit led to an 18.5-fold increase in maximal shedding (cohort mean) compared high-dose SR.

In contrast to the clear dose dependency, route of administration had subtler effects. Shedding prevalence was similar between IVT (4/4) and SR (5/6). Between these high-dose groups, copy numbers were moderately increased (2.7-fold) in subretinally injected animals, while shedding above LLOQ lasted 2 days longer after IVT. Individual results are displayed in Figure 4 and copy numbers/mL are given in Supplementary Material S1.

Transduction of the Visual System

At necropsy (91 days post injection), tissues of the visual pathway (Fig. 5; Table 2) were collected for formal biodistribution analyses. The segments of the visual system were sampled individually along the pathway taken by light (anterior segment, retina, optic nerve and chiasm, lateral geniculate nucleus [LGN], and visual cortex). Presence of vector in the aqueous humor was exclusively dependent on route of administration, while upstream distribution along the visual pathway was strongly associated with both dose and route of administration.

Ninety-one days after surgery, only one aqueous humor sample across both subretinal cohorts exhibited vector presence, while all samples in the intravitreal cohort were clearly above LLOQ. This was reversed within the eye, where compared to IVT, subretinal application of the same dose
resulted in 53 times higher copy numbers in whole retinal sections. A 10-fold dose escalation in SR caused a 37-fold increase of copy numbers detected in retinal tissue. The respective presence of vector in the retina carried over to following stations along the visual pathway. In optic nerve samples, dose escalation led to a 20-fold increase in copy numbers, while compared to IVT, SR resulted in 2.9-fold increased copy numbers. In line with the difference in copy numbers, five of six animals demonstrated vector presence in optic nerve samples after subretinal high-dose injections, as opposed to two of six animals in the low-dose cohort. A similar pattern, although with markedly lower copy numbers, also carried over to the optic chiasm, LGN, and visual cortex. Only one sample in the study (high-dose SR cohort) tested above the LLOQ for AH = $2.2 \times 10^4$ copies/mL sample.

The study also analyzed samples from blood (Figs. 1, 6; Table 1), major organs, and draining lymphatic tissue. Biodistribution in the systemic circulation and lymphatic tissues was strikingly dependent on the route of administration, but barely dose dependent. For example, while dosage had a moderate effect on biodistribution via blood, injecting intravitreally resulted in a 464-fold increased blood vector load compared to SR of the same dose, and vector presence in blood persisted in all animals of the IVT cohort until 91 days. Individual results are displayed in Figure 6 and copy numbers/mL are given in Supplementary Material S1.

### Large Organs and Lymphatic Tissue

In line with elevated vector load in blood after IVT, draining lymph node and spleen contained significant amounts of vector genomes in animals of the IVT cohort until 91 days. Individual results are displayed in Figure 6 and copy numbers/mL are given in Supplementary Material S1.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Aqueous Humor</th>
<th>Retina</th>
<th>Optic Nerve</th>
<th>Optic Chiasm</th>
<th>LGN</th>
<th>Visual Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-dose SR</td>
<td>0</td>
<td>$1.3 \times 10^6$</td>
<td>$1.2 \times 10^2$</td>
<td>$&lt;50$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>High-dose SR</td>
<td>$&lt;50$</td>
<td>$4.8 \times 10^7$</td>
<td>$2.4 \times 10^3$</td>
<td>$9.6 \times 10^2$</td>
<td>$&lt;50$</td>
<td>0</td>
</tr>
<tr>
<td>High-dose IVT</td>
<td>$3.2 \times 10^5$</td>
<td>$9.1 \times 10^5$</td>
<td>$8.4 \times 10^2$</td>
<td>$&lt;50$</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

On day 91, relevant copy numbers were detected only in the aqueous humor of IVT animals. Vector deposition in the retina and upstream the visual pathway was most pronounced in high-dose SR animals.
retropharyngeal, 510-fold for mandibular, and 490-fold for mesenteric lymph nodes, compared to SR. IVT led to an approximately 7400-fold increase in copy numbers in spleen compared to SR. Liver samples were also positive in the intravitreal cohort, while heart, tongue, spinal cord, adrenal glands, and, importantly, the gonads were free of vector genomes in all animals of all cohorts (Table 4).

**Summary**

The study was designed to explore the shedding and biodistribution characteristics after subretinal delivery of AAV8 when mimicking the clinical scenario in patients. The IVT control group was added to test effects of inadvertent primary injection into the vitreous (via falsa) and/or a secondary delivery into the vitreous cavity through a retinal tear (e.g., macular hole formation) in the context of subretinal delivery. Both dose and route of delivery change the distribution profile, and Figure 9 shows a proposed model based on the major finding that intravitreal placement of AAV8 results in much higher viremia and contact with immune-competent cells. It highlights the potential of immune-competent effector cells to influence local reactions to the viral vector in the eye.

**DISCUSSION**

To the best of our knowledge, this study reports the most comprehensive data on viral shedding and biodistribution of recombinant adeno-associated virus serotype 8 (rAAV8) after subretinal versus intravitreal injection (IVT) in nonhuman primates. This study was conducted in strict adherence to GLP guidelines and designed to generate data on biodistribution/shedding after subretinal and IVT in a protocol mimicking clinical application of rAAV8 gene therapy as closely as possible. In contrast to the protocol of the clinical trial, sclerotomies were not sutured in primates with the aim to reduce postsurgical irritation. However, this did not prevent digital manipulation of the treated eyes by the animals once transferred back to the cages. This may have affected biodistribution/shedding outcomes, and any such manipulation can easily be avoided in the clinical setting.

One important component of patient safety is avoidance of germ line transduction.13,14 There was no vector presence in the germ line, regardless of sex, vector dosage, or route of administration. Shedding of GMO into the environment is another important aspect, and our data show that shedding to the environment receded below LLOQ by 1 week after surgery, with 21% of samples without detectable vector

| Table 3. Vector Deposition in Lymph Nodes Given as vg per Organ After 91 Days |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cohort          | Deep Cervical   | Retropharyngeal | Mandibular      | Mesenteric      |                  |                  |                  |
| Low-dose SR     | 2.5 E+3         | 2.1 E+3         | 1.0 E+3         | 1.5 E+3         |                  |                  |                  |
| High-dose SR    | 8.1 E+3         | 2.3 E+3         | 7.0 E+3         | 4.5 E+3         |                  |                  |                  |
| High-dose IVT   | 3.5 E+6         | 2.4 E+6         | 3.6 E+6         | 2.2 E+6         |                  |                  |                  |

At a similar dose, intravitreal delivery (bottom row) was associated with pronounced deposition in lymph nodes close to and far from the eye, compared to subretinal delivery. Assumed organ weight: 0.1 g per lymph node.

**Table 4. Vector Deposition in Large Organs Given as vg per Organ After 91 Days**

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Spleen</th>
<th>Liver</th>
<th>Lungs</th>
<th>Heart</th>
<th>Gonads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-dose SR</td>
<td>4.6 E+5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>High-dose SR</td>
<td>6.6 E+5</td>
<td>0</td>
<td>&lt;LLOQ</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>High-dose IVT</td>
<td>4.9 E+9</td>
<td>3.2 E+6</td>
<td>&lt;LLOQ</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Significant off-target transduction of spleen and liver is observed after intravitreal administration.
observed after gene therapy.16,17 Such a systemic visibility after IVT might help to explain any form of immune response after IVT.19

It is important to note, however, that the absolute risk of any clinically relevant systemic toxicity in a patient after a retinal tear during subretinal delivery or IVT delivery can still be managed effectively. This is evident from clinical trials, where much higher dosages of rAAV8 were delivered primarily into the systemic circulation without causing serious adverse events.20 However, these trials have shown that systemic exposure to significant numbers of any rAAV serotype can induce humoral and cellular immune response followed by directed removal of successfully transduced target cells by cytotoxic T lymphocytes.21

Previous studies in large animal models have demonstrated that rAAV8 gene therapy has a favorable safety profile22 and efficiently transduces photoreceptors,23,24 and that vector genome can be found along the visual pathway after subretinal delivery of rAAV.25,26 Our data are in line with these reports and show that dose and route of application are important determinants of the extent of this distribution pattern.27 While our data do not explain the mechanism of transduction along the visual pathway, they clearly show that both routes of application can lead to anterograde vector genome distribution along the axons of the ganglion cells up to the LGN. Intriguingly, this would indicate that subretinal rAAV8 does transduce ganglion cells. Alternative explanations would involve trans-synaptic mobility and/or vector solution traveling in the subretinal space to reach the optic nerve sheath and access to the cortex, for example, via cerebrospinal fluid. Both seem rather unlikely scenarios and are not supported by the fact that the visual cortex is free from vector genomes.

When taking all biodistribution and shedding data into consideration, we argue that SR offers the more favorable set of results. With constant improvements of subretinal surgery,36,37 which promise to improve our understanding of this global distribution, we can clearly state that SR is therefore the preferable procedure in most current ocular gene therapy scenarios. This may of course change with the advent of new viral vectors30–35 and improvements in intravitreal surgery,36,37 which promise to efficiently transduce cells of the outer retina after intravitreal delivery.

While these results were generated using rAAV8, there is reason to believe that the mechanics underlying shedding and biodistribution are also applicable to other AAV serotypes with similar tropism. As most transgene cassettes in classic gene augmentation strategies feature target cell–specific promoters, off-target transduction with nonintegrating vectors such as AAV can still be regarded as fairly safe—especially as the germ line is shown not to be affected. However, in approaches using either ubiquitous promoters or immunogenic bacterial enzymes (e.g., CRISPR-Cas9) to edit genomic DNA with less than 100% specificity, off-target transduction may be more of a concern.38

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References


**APPENDIX**

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