Chapter 6

Successful re-administration of adeno-associated virus (AAV) vectors: cross administration of AAV serotypes 5 and 1

Anna Majowicz and Valerie Ferreira

Introduction

Adeno-associated virus (AAV) vector based gene therapy has proven to be very promising for the treatment of numerous genetic and inflammatory disorders [1, 2]. It has to be considered that re-administration of the therapy might be necessary due to the possibility of decrease of AAV-mediated gene expression overtime as a result of the natural turnover of transduced cells. This point is particularly valid when considering the treatment of life-long diseases.

The major challenge to achieve a successful re-administration is the presence of circulating neutralizing antibodies (NAB) directed against AAV capsids which are developed after the first administration of AAV vectors. Those neutralizing antibodies that recognize viral capsid proteins do not allow repeated gene transfer with AAV of the same serotype [3, 4, 5] Therefore, strategies that would permit a repeated gene delivery need to be developed.

In order to avoid this problem different AAV vector serotype with different antibody reactivity profiles and similar affinity to the target tissue could be used for the re-administration. Such serotype switching strategy is referred to as cross administration. Cross administration of AAV serotypes for the delivery of therapeutic protein has a great advantage over the alternative approach that involves use of immunosuppressive regimens. The immunosuppression strategy can lead to serious side effects and might not be applicable in all clinical settings depending on patient treatment history and health condition. It has been reported that AAV serotypes 1 and 5 could be used sequentially for re-administration in the muscle as no significant inhibitory cross-reaction were reported *in vivo* in muscle and *in vitro* in hepatic cell lines [3, 6]. In the present study we explored *in vivo* the efficacy of sequential intravenous administration of AAV serotype 5 and 1 for AAV re-administration in the liver.

Material and Methods

Ethics statement

All animal experiments were approved by the local animal welfare committee (University of Amsterdam).

AAV production

The AAV vectors batches (AAV5-hAAT-SEAP, AAV5-LP1-hFIX, AAV1-hAAT-eGFP and AAV1-LP1-hFIX) were produced in insect cells according to a technology adapted from R. M. Kotin [7]. The AAV vector batches were purified with an AVB sepharose column using the ÄKTA explorer system (GE Healthcare). Diafiltration and concentration of the AAV elution in PBS-/-, 5% sucrose buffer was performed with the use of hollow fiber membrane (Spectrum labs). The titer of AAV vector genomes copies (gc/ml) in the final product was determined by Taqman QPCR amplification.

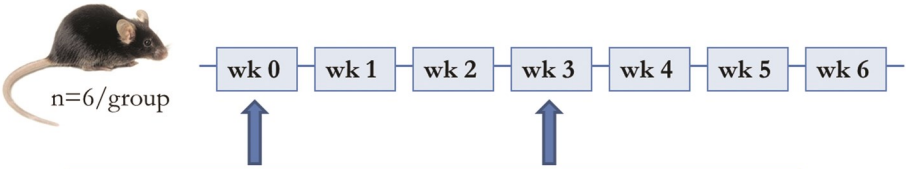
Animal Experiments

Male C57BL/6 mice (8-10 weeks) were obtained from Harlan and maintained in specific pathogen-free conditions at the animal facility.

In the first experiment mice (n=6/group) were injected intravenously (iv) with AAV5-hAAT-SEAP or PBS at week 0. The second intravenous administration with AAV1-LP1-hFIX, AAV1-hAAT-eGFP or PBS was performed at week 3 (**Figure 1**).

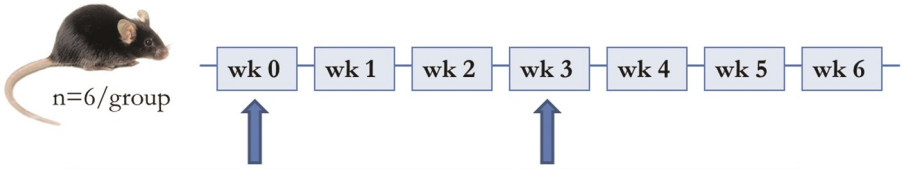
In the second experiment mice (n=6/group) were injected intravenously (iv) with AAV5-hAAT-SEAP or PBS at week 0. The second intravenous administration with AAV1-LP1-hFIX, AAV5-LP1-hFIX or PBS was performed at week 3 (**Figure 2**). The dose of all the AAV batches that were injected was 1.46×10^{13}gc/kg and injection volume was 10 µl/g.

For both experiments, blood was collected weekly by submandibular vein puncture in tubes containing sodium citrate. Plasma was isolated after centrifugation for 20 min at 2500 g at 4°C and stored at -80°C until further analysis. All mice were sacrificed at week 7. Liver tissues were collected and snap frozen in liquid nitrogen with or without pre-fixation in picric acid and stored at -80°C until further analysis.



Groups	1st intravenous injection	2nd intravenous injection
1.	PBS	PBS
2.	PBS	AAV1-LP1-hFIX
3.	PBS	AAV1-hAAT-eGFP
4.	AAV5-hAAT-SEAP	PBS
5.	AAV5-hAAT-SEAP	AAV1-LP1-hFIX
6.	AAV5-hAAT-SEAP	AAV1-hAAT-eGFP

Figure 1.
Scheme of the first experimental setup; n=6/group.



Groups	1st intravenous injection	2nd intravenous injection
1.	PBS	PBS
2.	PBS	AAV1-LP1-hFIX
3.	PBS	AAV5-LP1-hFIX
4.	AAV5-hAAT-SEAP	PBS
5.	AAV5-hAAT-SEAP	AAV5-LP1-hFIX
6.	AAV5-hAAT-SEAP	AAV1-LP1-hFIX

Figure 2.
Scheme of the second experimental setup; n=6/group.

Assessment of transgenes expression

Human FIX expression was measured in plasma of mice with the use of FIX ELISA kit (VisuaLize™ FIX Antigen Kit, Affinity Biologicals™$_{INC}$).

SEAP expression was measured in mouse plasma with the use of chemiluminescent "SEAP Reporter Gene Assay" (Roche).

GFP expression was assessed by post mortem fluorescent microscopy of liver tissue sections of mouse livers that were fixed in picric acid upon harvesting.

Assessment of anti-AAV5 and anti-AAV1 antibody levels

Levels of anti-AAV5 antibody in mouse plasma was measured by an anti-AAV5 specific ELISA. Practically, MaxiSorp® flat-bottom 96-well plates (ThermoScientific) were coated with AAV5 capsid and anti-AAV5 antibody level in samples was detected with 1:1000 rabbit anti-mouse Immunoglobulins/HRP (DAKO).

Level of anti-AAV1 antibody in mouse plasma was measured by anti-AAV1 specific ELISA. MaxiSorp® flat-bottom 96-well plates (ThermoScientific) were coated with AAV1 capsid and anti-AAV1 antibody level in samples was detected with 1:1000 rabbit anti-mouse Immunoglobulins/HRP (DAKO).

NAB assay against AAV5 and AAV1 capsid

HEK293T cells were seeded in 96-well plates (Corning) that were pre-coated with 0.25% poly L-lysine at a density of 1 x 10^5cells/well in 100 µl of DMEM with 10% FBS and 1% Penicillin/Streptomycin. Cells were incubated overnight at 37°C in 5% CO_2 water jacket incubator. Medium was then removed and the following mix was added: AAV5-CMV-GFP for anti-AAV5 NAB assay or AAV1-CMV-GFP for anti-AAV5 NAB assay with heat-inactivated plasma sample in a total volume of 100 µl of DMEM without phenol red and 1% Penicillin/Streptomycin. The mix was incubated for 1 hour at 4°C prior to addition on the cells. Medium of the HEK293T cells was removed by aspiration, and then the mix was added and incubated for 16-20 h at 37°C. Serial dilutions of test plasma that were prepared were: 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, and 1:12800. As a positive control, cells without plas-

ma addition in the mix were analyzed. After 16-20 h, cells were rinsed with PBS, collected after trypsinization and fixed in PBS, 2% Formaldehyde, 1% BSA. GFP expression of the cells was analyzed by flow cytometry (FACScalibur, Becton Dickinson) in channel FL1 at a wavelength of 530 nm. The analysis was performed with the Cellquest Pro software. The percentage of inhibition was calculated related to GFP expression measured in AAV HEK293T infected cells (no inhibition, 100% expression). Plasma dilutions causing a 50% reduction of GFP expression when compared to positive control, were determined (EC50 determination after sigmoidal curve fit in GraphPad Prism software).

Results

No cross reactivity of antibodies raised against AAV5 and AAV1 capsids was observed.

Mice were injected intravenously (iv) with AAV5-hAAT-SEAP or PBS at week 0. The second intravenous administration with AAV1-LP1-hFIX, AAV5 -LP1-hFIX or PBS was performed at week 3. In order to determine the total anti-AAV antibody levels and anti-AAV neutralizing antibody (NAB) levels plasma samples of injected animals were analyzed.

Total anti-AAV5 antibodies were detected in the plasma of all the mice that were injected with AAV5 vectors, the levels of antibodies increased over time until week 3 and remained stable until the end of the experiment (AAV5-hAAT-SEAP and AAV5-LP1-hFIX) (**Figure 3.A**). Similarly, all the mice that were injected with AAV1-LP1-hFIX developed antibodies against AAV1 with an increase until week 3 after injection was performed (**Figure 3.B**). As expected, anti-AAV1 antibodies were not detected in plasma of mice that were injected with AAV5-hAAT-SEAP alone, AAV5-LP1-hFIX alone or PBS (**Figure 3.B**) as well as anti-AAV5 antibodies were not detected in plasma of mice that were injected with AAV1-LP1-hFIX alone or PBS (**Figure 3.A**).

Overall, those results demonstrate the absence of cross reactivity between total AAV5 and AAV1 antibodies in our assay. In case of two sequential injections with AAV5-based vectors, a low level of recognition of AAV1 capsids

by anti-AAV5 antibodies was observed, which is probably due to general enhancement of immune system responses (**Figure 3.B**).

In order to determine the potential of the measured total antibodies to neutralize AAV transduction, neutralizing antibodies assays were performed for both AAV5 and AAV1 serotypes. The neutralizing antibody (NAB) titers against AAV5 capsid were similar in all the mice injected once with AAV5-hAAT-SEAP or AAV5-LP1-hFIX. The anti-AAV5 NAB titers were slightly higher in the animal group that was injected twice with AAV5 (AAV5-hAAT-SEAP followed by AAV5-LP1-hFIX) (**Figure 4.A**).

Similarly, neutralizing antibody (NAB) titer against AAV1 capsid raised in plasma of mice that were injected with AAV1-LP1-hFIX was measured. No NAB antibodies against AAV1 capsid were detected in the mice groups that were injected with AAV5-hAAT-SEAP alone, AAV5-LP1-hFIX alone or PBS (**Figure 4.B**).

Our Results demonstrate the absence of cross reactivity between NAB against AAV5 and AAV1 capsids.

Stable dual gene expression after sequential intravenous administration of AAV5- and AAV1-mediated gene delivery

Mice were injected intravenously (iv) with AAV5-hAAT-SEAP or PBS at week 0. The second intravenous administration with AAV1-LP1-hFIX, AAV1 -hAAT-eGFP, AAV5-LP1-hFIX or PBS was performed at week 3. In order to determine the expression of SEAP and hFIX protein, plasma of injected animals was collected weekly, while the eGFP was assessed by post mortem fluorescent microscopy of liver tissue sections.

SEAP expression was detected in the plasma of all the mice injected with AAV5-hAAT-SEAP (**Figure 5.A, 6.A**). This expression increased from week 0 to week 2 and was stable until sacrifice at week 7. The mice that received AAV1-LP1-hFIX in second injection at week 3 showed raising expression of hFIX protein in plasma from week 4 which stabilized at week 5 and remained stable until sacrifice. At the opposite, the mice which received AAV5-LP1-hFIX in second injection did not express detectable level of FIX in the plasma

(**Figure 5.B**). It should be noted that the expression level of hFIX protein that appeared after injection with AAV1-LP1-hFIX was not influenced by the prior injection with AAV5-hAAT-SEAP as the same level of FIX was measured in the control group that received PBS in first injection and AAV1-LP1-hFIX in second injection (**Figure 5.B.**).

The expression level of hFIX protein in plasma of mice injected with AAV5-LP1-hFIX was higher than in plasma of mice injected with AAV1-LP1-hFIX. This observation confirms previous publications reporting that AAV5 transduces the liver more efficiently than AAV1. However, the level of hFIX protein measured in the mouse plasma after delivery with AAV1 vector is above therapeutic level which indicates that AAV1 serotype could also be efficient for gene delivery in the liver.

Successful cross administration of AAV5 and AAV1 vector was also achieved with combination of others expression cassettes: AAV5-hAAT-SEAP and AAV1-hAAT-eGFP (week 3) when both SEAP (**Figure 6.A**) and eGFP (**Figure 6.B.III**) were expressed by the injected animals.

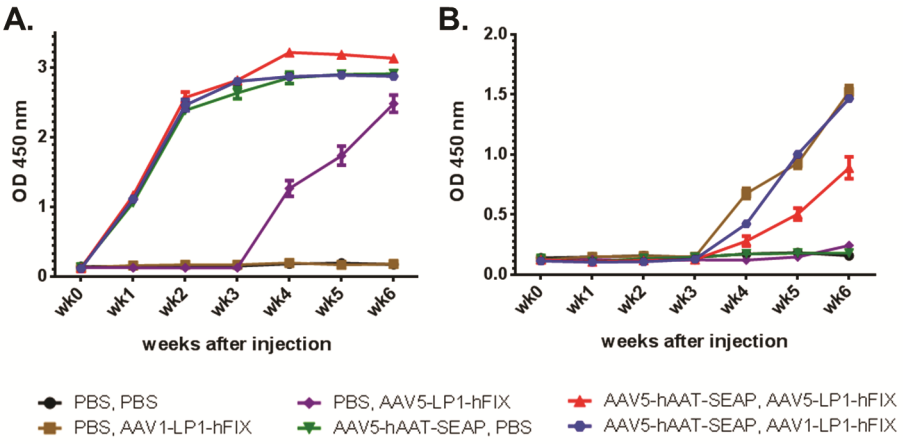


Figure 3. Total anti-AAV5 (A) and anti-AAV1 (B) antibody level in mouse plasma. Mice received intravenous injections of PBS or AAV5-hAAT-SEAP at week 0 and second intravenous injection of PBS, AAV1-LP1-FIX or AAV5-LP1-FIX at week 3; n=6/group.

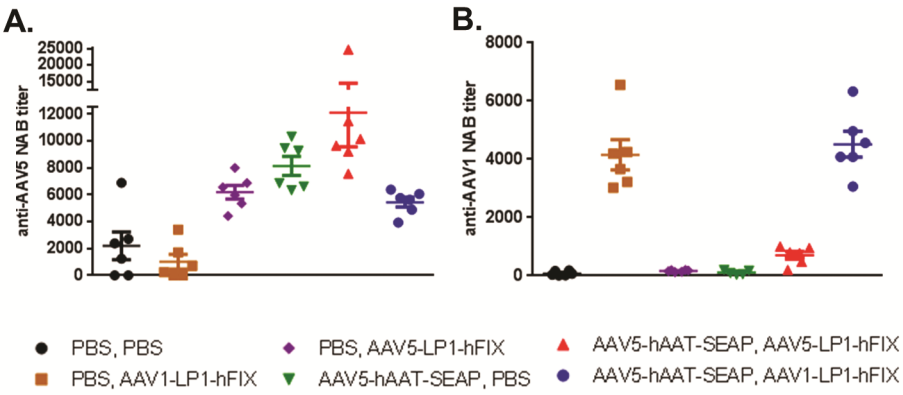


Figure 4. Anti-AAV5 (A) and anti-AAV1 (B) NAB level in mouse plasma at week 6. Mice received intravenous injections of PBS or AAV5-hAAT-SEAP at week 0 and second intravenous injection of PBS, AAV1-LP1-FIX or AAV5-LP1-FIX at week 3; n=6/group.

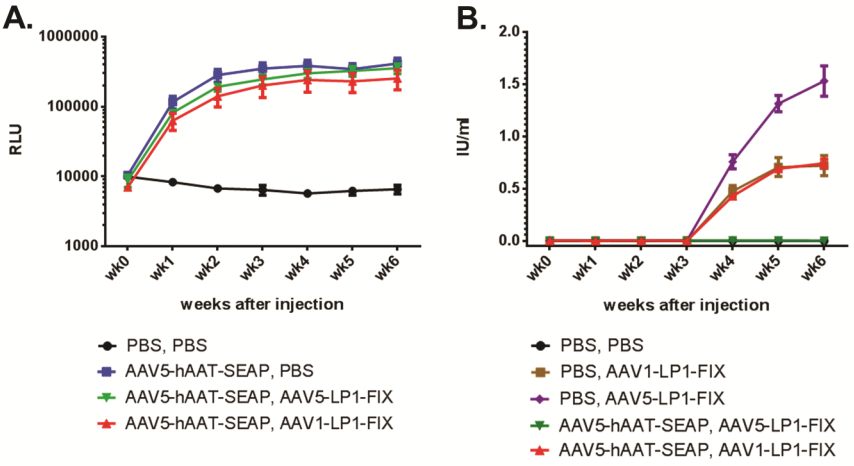


Figure 5. Expression of SEAP (A) and FIX protein (B) in mouse plasma overtime.
Mice received intravenous injections of PBS or AAV5-hAAT-SEAP at week 0 and second intravenous injection of PBS, AAV1-LP1-FIX or AAV5-LP1-FIX at week 3; n=6/group.

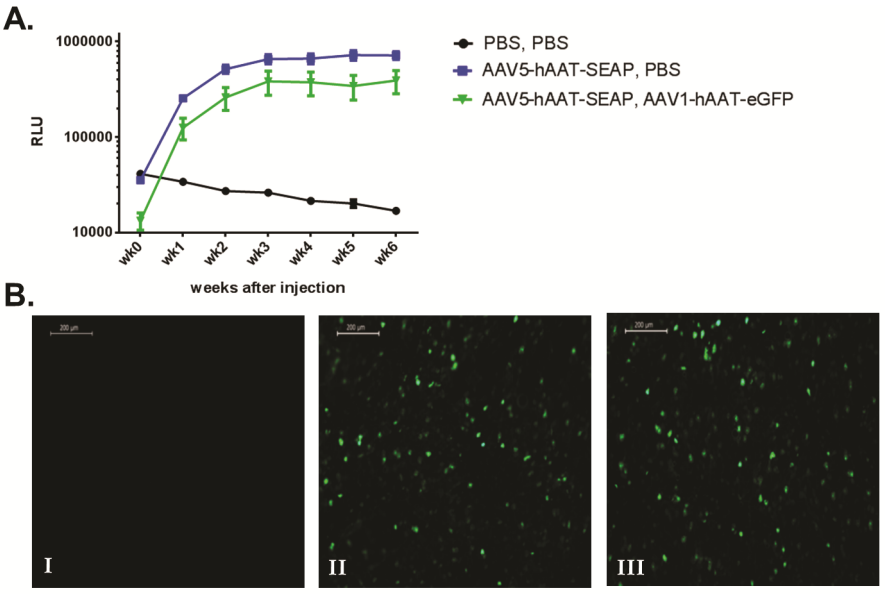


Figure 6. Expression of SEAP measured overtime in mouse plasma (A) and expression of eGFP in representative mouse liver sections (B).
Mice received intravenous injections of PBS or AAV5-hAAT-SEAP at week 0 and second intravenous injection of PBS or AAV1-hAAT-eGFP at week 3; n=6/group. B: **I**- PBS, PB group; **II**- PBS, AAV1-hAAT-eGFP group; **III**- AAV5-hAAT-SEAP, AAV1-hAAT-eGFP group; RLU- Relative Luminescence Units.

Discussion

The study presented in this chapter demonstrates the feasibility of re-administration of AAV-based vectors when using sequentially the serotypes 5 and 1 for liver targeting. We have shown that the anti-AAV neutralizing antibodies (NAB) were not cross reactive between the serotypes 5 and 1 and that a high level of gene expression was observed after sequential delivery of reporter genes from both AAV5 and AAV1 vectors. In contrast, the re-administration of the same serotype (AAV5) was unsuccessful due to the total inhibition of secondary AAV5 transduction by anti-AAV5 NAB. Our data confirms the previously reported *in vitro* study where the cross-reactivity and neutralization mechanisms between AAV1 and AAV5 serotypes were examined and no cross-reactivity between them was demonstrated [5].

The cross administration approach with AAV5 and AAV1 appears to be efficient for hepatic gene transfer as a high level of gene expression was measured for both reporter genes used (SEAP and hFIX). It should also be noted that despite the fact that hFIX protein expression level after delivery by the AAV1 serotype is lower than after delivery with AAV5 vector, it remains above the defined therapeutic level. All together, these data indicate that AAV1 serotype is also a good candidate for liver targeting. However, further evaluation of the efficiency of AAV5 and AAV1 cross administration for liver targeting is necessary and non-human primate studies need to be performed in order to evaluate the future clinical relevance of such approach. Despite the fact that this approach has proven to be successful and could be a good option for many patients, changing serotypes, also changes the drug from regulatory point of view and that implies separate costly drug approval pathways for every serotype used and that certainly is a limiting factor.

REFERENCES

1. Nathwani, AC, Tuddenham, EG, Rangarajan, S, Rosales, C, McIntosh, J, Linch, DC, et al. (2011). Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med*; 365: 2357-2365.

2. Gaudet, D, Methot, J, Dery, S, Brisson, D, Essiembre, C, Tremblay, G, et al. (2013). Efficacy and long -term safety of alipogene tiparvovec (AAV1-LPL(S447X)) gene therapy for lipoprotein lipase deficiency: an open-label trial. *Gene Ther*; 20: 361-369.

3. Riviere, C, Danos, O and Douar, AM (2006). Long-term expression and repeated administration of AAV type 1, 2 and 5 vectors in skeletal muscle of immunocompetent adult mice. *Gene Ther*; 13: 1300-1308.

4. Nathwani, AC, Davidoff, A, Hanawa, H, Zhou, JF, Vanin, EF and Nienhuis, AW (2001). Factors influencing in vivo transduction by recombinant adeno-associated viral vectors expressing the human factor IX cDNA. *Blood*; 97: 1258-1265.

5. Harbison, CE, Weichert, WS, Gurda, BL, Chiorini, JA, Agbandje-McKenna, M and Parrish, CR (2012). Examining the cross-reactivity and neutralization mechanisms of a panel of mAbs against adeno-associated virus serotypes 1 and 5. *J Gen Virol*; 93: 347-355.

6. Grimm, D, Zhou, S, Nakai, H, Thomas, CE, Storm, TA, Fuess, S, et al. (2003). Preclinical in vivo evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. *Blood*; 102: 2412-2419.

7. Urabe, M, Ding, C and Kotin, RM (2002). Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum Gene Ther*; 13: 1935-1943.