Gene therapy with adeno-associated viral vectors for inherited hyperbilirubinemia: towards a clinical trial for Crigler-Najjar syndrome

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Gender bias in AAV liver transduction

Manuscript in preparation

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Abstract

Recent gene therapy trials have demonstrated clinical efficacy in patients suffering from various inherited disorders. The central role of the liver in metabolic processes renders it an important target for gene therapy. We showed previously that in vivo gene therapy for inherited liver disorders seems feasible. Using a single-stranded adeno associated Viral vector (AAV) we obtained life long correction of unconjugated hyperbilirubinemia in male Gunn rats, the relevant animal model for Crigler-Najjar syndrome. In the meantime, however, several studies reported that liver transduction by ssAAV vectors is less efficient in females of animal models such as mice and wood-chucks. A large difference in efficacy between genders will limit the clinical use of AAV as a vector when aiming to treat an autosomal disorder like CN syndrome. To establish an efficient treatment in both genders we developed a self-complementary AAV (scAAV) vector, previously reported to be efficient in females of other animal models. We demonstrate that a dose of $3 \times 10^{11}$ gc/kg of this novel vector is therapeutic in both genders, furthermore in a liver-directed AAV clinical trial a comparable vector dose showed to be safe and without induction of liver damage. The ssAAV vector, in contrast, was only effective in male rats. Conclusion: We developed a liver specific gene therapy vector that, at a low dose, provides complete correction of unconjugated hyperbilirubinemia in both genders of Gunn rats.
Introduction

Crigler-Najjar (CN) syndrome type 1 is an autosomal recessive inherited liver disorder caused by deficiency of uridine diphospho-glucuronosyl transferase 1A1 (UGT1A1)\(^1,2\). UGT1A1 catalyzes the glucuronidation of unconjugated bilirubin, an essential step in excretion of this neurotoxic compound into bile. As a result, CN type 1 patients accumulate unconjugated bilirubin and are at risk of bilirubin encephalopathy (kernicterus)\(^3\). Currently, these patients are treated with phototherapy. Although effective during childhood, efficacy decreases with age and eventually most CN type 1 patients do need a liver transplantation\(^4\). The burden of long-lasting phototherapy, the limited availability of donor organs and the side-effects of life-long immunosuppression after liver transplantation, warrant the development of alternative treatments such as gene therapy.

We previously showed that liver directed gene therapy using vectors derived from the single stranded adeno-associated virus serotype 1 (AAV1) seems feasible for CN syndrome. A single injection of a single stranded AAV1 vector (ssAAV1) encoding human UGT1A1 provided a life-long therapeutic correction of serum bilirubin levels in male Gunn rats\(^5\). In the meantime, however, several studies reported that liver transduction by ssAAV vectors is less efficient in females of animal models such as mice and woodchucks\(^6,7\). A large difference in efficacy between genders will limit the clinical use of AAV as a vector when aiming to treat an autosomal disorder like CN syndrome. The mechanism underlining the gender difference in ssAAV liver transduction efficiency is not fully understood. But, it has been hypothesized that an androgen dependent interaction between vector genome and nuclear proteins may play a role in liver cell transduction\(^7\).

A comparable efficacy in both genders is an important pre-requisite for clinical application of AAV mediated gene therapy in autosomal disorders such as CN syndrome. Therefore we compared the efficacy of our liver specific ssAAV1 vector in male and female Gunn rats and found it to be much less effective in female rats. To establish a standardized treatment for both genders we decided to construct a so called self complementary (sc) AAV1 vector. This vector contains a double stranded DNA genome and is expected to overcome the poor efficacy of ssAAV1 vector in females. In this study, we show that a single portal vein injection of our scAAV1 vector provided a life-long correction of the hyperbilirubinemia in both male and female Gunn rats.
Material and Methods

Construction and production of AAV vectors. The ssAAV1-L-FABP-UGT1A1 vector was constructed by replacing the CMV promoter from the pTRCGW-UGT1A1 AAV vector by the proximal 620 bp promoter of liver fatty acid binding protein 1 (L-FABP). The scAAV1-LP1-UGT1A1 was constructed by replacing the factor IX cDNA with the UGT1A1 cDNA, using the EcoR1 and Bbs1 sites of plasmid scAAV-LP1-hFIXco. Both vectors were sequenced to confirm proper insertion of UGT1A1. Recombinant AAV1 vectors were produced and purified as described in. Titration of AAV1 vectors was performed by quantitative PCR. The scAAV1 vector was considered as containing 2 copies of the genome.

Analysis of genomic DNA and mRNA. High-molecular-weight DNA from tissues was isolated according to Boom et al. Real time quantitative PCR (qPCR), to determine the genomic copy number of UGT1A1 normalized to β-actin expression in 100ng of genomic DNA, was performed in Roche LightCycler480 using fast-start SYBRgreen kit. UGT1A1 primers: 5′-GACGCCTCGTTGATACATCAG-3′; 5′-CACGCTGCAGGAAAGAATC-3′ and rat β-actin primers: 5′-AGCCATGTACGTAGCCTCCA-3′; 5′-TCTCCGGAGTCTCATCAATG-3′. Dilutions of UGT1A1 plasmid into rat genomic DNA were used to generate copy number standards. To determine UGT1A1 expression, RNA was isolated using Trizol reagent (Invitrogen). cDNA was generated using Super-script3 RT (Invitrogen) and oligo(dT) primers and used for qPCR. The results were normalized to 18S rRNA. The LinRegPCR program was used to calculate the relative copy number detected by qPCR.

Animal experiments. All animal experiments were performed in accordance with the Animal Ethical Committee guidelines of the Academic Medical Center of Amsterdam. Gunn rats from our own colony, 8-10 weeks of age, with a weight between 180 and 200g for males and between 100 to 120g for females, were fed ad libitum and randomly assigned to different treatment groups. Male rats received an intraportal injection of 3x10^{12}gc/kg of ssAAV1-L-FABP-UGT1A1 or 1x10^{11}gc/kg, 3x10^{11}gc/kg or 1x10^{12}gc/kg of scAAV1-LP1-UGT1A1. Female rats received an intraportal injection of 3x10^{12}gc/kg of ssAAV1-L-FABP-UGT1A1 or 3x10^{11}gc/kg of scAAV1-LP1-UGT1A1. Portal injections, blood sampling and bile collection were performed as described.

Liver immunohistochemistry. Liver specimens were fixed in 4%PFA in PBS and embedded in paraffin. Sections (6-μm) were deparaffinized with xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase activity was blocked with
3%\textsubscript{H\textsubscript{2}O\textsubscript{2}} in PBS. Non specific binding was prevented by incubation with an avidin and biotin blocking solution (Vector Laboratories, Burlingame, CA). Tissues sections were incubated overnight with P6215, a polyclonal rabbit antiserum against human UDPGTs (1:10000)\textsuperscript{11}. Next, the sections were incubated with a biotinylated goat anti-rabbit IgG (1:400) for 10’, followed by a 10’ incubation with a LSAB2 Streptavidin-HRP (DAKO, K1016) and HRP detection with Vector NovaRED substrate (Brunschwig, SK-4800). Hematoxylin was used as nuclear counter stain.

**Bilirubin quantification.** Total bilirubin in serum was determined by the routine clinical chemistry department using a standard colorimetric assay. Unconjugated bilirubin and bilirubin conjugates in bile were quantified by HPLC using an Omnisphere column (Varian, The Netherlands) as described\textsuperscript{5,12}.

**Results**

**Reduced liver transduction efficiency of single stranded AAV in female Gunn rats**

Intraportal injection of a dose of 3\times10^{12}\textsubscript{gc/kg} of ssAAV1-L-FABP-UGT1A1 provided a significant correction of serum bilirubin levels in males (Fig.1A, p<0.01) while in females this dose was not effective (Fig.1B). The low levels of UGT1A1 mRNA (Fig.1C, p<0.01) and AAV genomic copies detected in liver confirm the poor efficacy in females (Fig.1D, p<0.001,Table1). Also the lower percentage bilirubin glucuronides in bile of females (~50%) compared to the ~90% found in males confirms a lower bilirubin glucuronidation activity in females (Table1, p<0.001).

**Table 1. Bile composition confirms functional ssAAV1-LFABP-UGT1A1 Gene Transfer.** The percentage of unconjugated (%UCB), monoglucuronidated (%MCB) and diglucuronidated (%DCB) bilirubin in bile was determined by HPLC. The number of AAV transgene copies in liver was determined by qPCR using β-actin as a reference gene. All studies were performed at 62 weeks after vector administration. Data presented as means ±SD. ND (Not Detectable). (#) (p<0.001) and (*) (p<0.05), compared between gender.

<table>
<thead>
<tr>
<th>Animal gender</th>
<th>n\textsuperscript{o} of transgene copies / dGE</th>
<th>Bile composition</th>
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<tr>
<td></td>
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<td>%DCB</td>
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<td>Female(n=4)</td>
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Fig. 1 – Correction of serum bilirubin levels in male but not in female Gunn rats by liver specific ssAAV1. Gunn rats intraportally injected with 3x10^{12} gc/kg ssAAV1-L-FABP-UGT1A1. (A) Total serum bilirubin levels were measured in untreated (◇, n=15) and in injected (◇, n=5) male rats. (B) Total serum bilirubin levels were measured in untreated (○, n=4) and in injected (●, n=3) female rats. Data represent the mean and standard deviation per time point. (C) Relative UGT1A1 mRNA levels in the liver of female (●, n=3) and male (▲, n=5) rats at 62 weeks after injection was determined by qPCR and normalized for 18S rRNA. (D) UGT1A1 transgene copy number per diploid genome equivalent (dGE) in livers of female (●, n=3) and male (▲, n=5) animals at 62 weeks after injection were calculated using the ratio of UGT1A1 and rat β-actin gene copies in 100 ng of DNA as quantified by qPCR. ND (Not Detectable). Statistical significant differences where determined by Student (unpaired) t test.

Comparable liver transduction efficiency by scAAV in both Gunn rats genders

The poor efficacy of ssAAV1 renders this vector inapplicable in female patients. To overcome this we generated a liver specific double stranded vector, scAAV1-LP1-UGT1A1. In contrast to the ssAAV1 vector this scAAV1 vector did provide efficient correction in both female and male Gunn rats. In both genders a single injection of 3x10^{11} gc/kg normalized serum bilirubin levels (Fig. 2A, p<0.05). The similar efficiency in both genders is also reflected by comparable UGT1A1 mRNA levels and AAV genomic copy numbers (Fig. 2 B, C; Table 2). However, the reduction of serum bilirubin levels in males is still more pronounced, which is also reflected by a higher percentage of bilirubin glucuronides in bile (Table 2).
In addition, we determined the efficacy of a three-fold higher and lower dose in male Gunn rats. This showed that even the low dose of $1 \times 10^{11}$ gc/kg provides a 60% reduction of serum bilirubin levels and presence of $\sim$90% of bilirubin glucuronides in bile (Table 2), while the highest dose normalizes the levels. UGT1A1 mRNA levels, the number of AAV genomic copies in the liver (data not shown) and the percentage of UGT1A1 expressing hepatocytes (data not shown) all correlate with the administered vector dose. In summary, a dose of $3 \times 10^{11}$ gc/kg of scAAV1-LP1-UGT1A1 provides therapeutic reduction of unconjugated hyperbilirubinemia both in male and female Gunn rats.
Table 2. Bile composition and Immunohistochemistry confirm functional scAAV1-LP1-UGT1A1 Gene Transfer. The percentage of unconjugated (%UCB), monoglucuronidated (%MCB) and diglucuronidated (%DCB) bilirubin in bile was determined by HPLC. The number of AAV transgene copies in liver was determined by qPCR using β-actin as a reference gene. All studies were performed at 62 weeks after vector administration. Data presented as means ±SD. ND (Not Detectable). (#) (p<0.001) and (*) (p<0.05), compared between gender.

<table>
<thead>
<tr>
<th>Animal gender</th>
<th>Dose (gc/kg)</th>
<th>n⁰ of transgene copies / dGE</th>
<th>Bile composition</th>
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<td>0.4±0.1</td>
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Fig.3 – Presence of UGT1A1 expressing hepatocytes in the liver of AAV1 treated Gunn rats. Representative sections of the different groups are shown: male and female rats injected with 3x10¹² gc/kg ssAAV1-L-FABP-UGT1A1 or with 3x10¹¹ of scAAV1-LP1-UGT1A1.

Biodistribution

AAV1 transduction is not limited to the liver. We previously showed transduction of organs such as lung, kidney, heart and spleen⁵. To investigate if AAV1 biodistribution is affected by gender we determined the presence of AAV genomes in these organs.
and in the testis and ovaries. The ratio between UGT1A1 and the endogenous rat β-actin gene was used to calculate the number of AAV genomes per diploid genome equivalent (dGE). Although we could detect AAV genomes in all tested organs the levels were at least 100 fold lower compared to liver (Fig.4). No gender-related effect was seen on vector biodistribution in these tissues.

![Fig.4 — Biodistribution of scAAV1-LP1-UGT1A1 upon portal vein injection in Gunn rats. UGT1A1 transgene copy number per diploid genome equivalent (dGE) in the liver at 62 weeks after injection of 3x10^{11} gc/kg of scAAV1-LP1-UGT1A1 of female (white bars) and male (black bars) Gunn rats. DNA was isolated from liver, lung, testis/ovaries, spleen, heart and kidney. The UGT1A1 copy number was calculated using the ratio of UGT1A1 and the rat β-actin gene determined by qPCR in 100 ng of DNA.](image)

**Discussion**

In the relevant animal model, the Gunn rat, we previously demonstrated that AAV-mediated gene therapy for Crigler-Najjar syndrome seems feasible at least in males\(^5\). Since CN syndrome is an autosomal disease, we investigated the efficacy of this approach in female Gunn rats. Compared to males, liver transduction with the ssAAV1-L-FABP-UGT1A1 vector is less efficient in female rats, based on a poor correction of serum bilirubin levels, a low AAV transgene copy number and low UGT1A1 mRNA levels in the liver. Our data in rats confirm those seen in mice and woodchucks\(^6,7\). Presence of a gender effect in different animal models indicates that it is a general phenomenon and therefore is relevant when aiming to treat female patients suffering from an inherited liver disorder with ssAAV1.

The gender difference in liver transduction efficiency in Gunn rats is minimized when using a scAAV1 vector, where both genders have comparable mRNA levels and genomic copies of UGT1A1. This observation supports the hypothesis that androgen
dependent interactions between host hepatocellular nuclear proteins and the AAV genome are important for the second-strand synthesis\textsuperscript{7}. However, the 60% reduction in serum bilirubin levels in females compared to the 80% seen in males suggests that additional steps may be impaired (Fig.2A). Pañeda \textit{et al.} also showed that using scAAV1 in mice did not entirely overcome the difference in liver transduction between genders\textsuperscript{13}. One explanation for this residual difference could be that the stabilization of the episomal form, is impaired in females. However, the comparable transgene copy numbers in liver of both genders after a year suggest that this is unlikely. Another possibility, as proposed by Osman \textit{et al.}, is a lower transcriptional activity of the LP1 promoter in females\textsuperscript{14}. In fact, the transcriptional activity of the alpha-1-trypsin promoter, which is a main constituent of LP1 promoter, is affected by gender\textsuperscript{15}. However comparable UGT1A1 mRNA levels in liver indicate this is unlikely, suggesting that such an effect will be small if present at all. In the liver comparable transgene copy numbers and mRNA levels suggest that the residual gender difference seems due to a post-transcriptional impairment. In this respect the findings of Laz \textit{et al.} are of interest. They showed that differential secretion patterns of growth hormone contribute to the sexual dimorphism of the nuclear proteomic profile, especially in rodents\textsuperscript{16}. This may have sex-dependent effects on stability, localization of proteins and post-translational modifications\textsuperscript{16}. Such a mechanism may explain the higher percentage of biliary bilirubin glucuronides indicating an increased bilirubin glucuronidating activity in male livers, while transgene copy number and mRNA levels are similar to those in female Gunn rats.

Nevertheless, our study shows that the difference in efficiency between male and female rats is minimized when using $3 \times 10^{11}$ gc/kg of scAAV1-LP1-UGT1A1 vector. The therapeutic correction of serum bilirubin levels with this dose warrants clinical application in male and female CN patients.

\textbf{Acknowledgements}

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References


