Development of a lead candidate for Shire's AAV8-based FVIII gene therapy program BAX 888

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INTRODUCTION

Adeno-associated virus (AAV)-based gene therapy holds great promise for the treatment of hemophilia, since data from several phase 1/2 clinical trials in patients with hemophilia B indicated that therapeutically relevant FIX levels can be achieved for an extended period of time. These studies also revealed the importance of developing high-expressing vectors that enable sufficient expression at doses at which the AAV-associated immune response remains manageable. In hemophilia A, developing efficient transgene expression cassettes is even more relevant in light of the many challenges associated with FVIII expression. Not only does the large size of the FVIII coding sequence preclude packaging of full-length FVIII into AAV, but it is also known to be poorly translated and secreted.

(For detailed information on the biopotency of the construct, please see Hoellriegl et al., Biopotency and efficacy of a FVIII gene therapy construct in hemophilia A mice, P-M-32).

OBJECTIVE

To design virus vectors based on the AAV8 serotype, a single-stranded genome architecture, and expression cassettes that confer liver-specific expression of a B-domain deleted Refacto-type variant of FVIII.

METHODS

Plasmids were constructed by cloning different synthetic DNA fragments, encoding FVIII into an AAV expression cassette (Chatham 47) that was flanked by AAV2 inverted terminal repeats (ITRs). The cDNAs were preceded by a promoter/ enhancer sequence derived from the liver-specific murine transthyretin gene. DNA synthesis of the Refacto-type BDD-FVIII fragments with flanking *Asc*I and *Not*I enzyme restriction sites was done by ThermoFisher Scientific (Regensburg, Germany). A total of 44 different AAV constructs with FVIII coding sequence variants were cloned and packaged.

AAV8-based vectors were prepared by the three plasmid transfection method as described (1). HEK293 suspension cells were used for plasmid transfection using the corresponding FVIII vector plasmid, the helper plasmid pXX6-80 (carrying adenoviral helper genes), and the packaging plasmid pGSK2/8 (contributing the rep2 and cap8 genes). The cell pellets of 1 liter cultures were processed using iodixanol gradients followed by a one-step purification (1). Vectors were quantified by qPCR (2).

Vector integrity assay. The integrity of the vector genomes was analyzed using AAV agarose gel electrophoresis. The procedure was performed as described (3). Approximately 1.5E+10 vector genomes (vg) were loaded per lane.

In vivo biopotency assay. Male FVIII knock-out (ko) mice (n=6-8 per group) received a single intravenous dose of 4E+12 vector genomes (vg) per kg body weight. Blood samples were taken every week over 4 weeks and analyzed for FVIII activity using a chromogenic activity assay (Technochrome FVIII assay; Technoclone, Vienna, Austria). FVIII expression levels at day 14 were chosen for screening, because at this time point, the influence of inhibitory antibodies was minimal.

RESULTS

Construction of a codon-altered factor VIII variant expression sequence

- Genomic size limitations of AAV gene therapy vectors only allow expression of B-domain deleted Factor VIII (FVIII-BDD) variants. We chose the 'FVIII-BDD-SQ' variant, whose B-domain is replaced with a 14 amino acid 'SQ' sequence (**Figure 1**), as recombinant FVIII-BDD-SQ (REFACTO®) has been shown to be effective in managing hemophilia A.

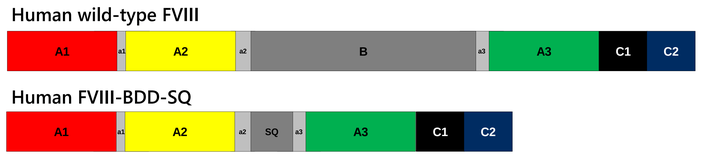

Figure 1: FVIII genes and vector construct

- A screening program for improved FVIII coding sequences was initiated that used a combination of available codon-optimization algorithms and manual sequence editing. Altogether 44 codon-optimized BDD FVIII sequences were packaged into AAV2/8 vectors and screened for FVIII expression. The performance of a selection of constructs is shown in **Table 1**.
- Codon-altered sequences using commercial algorithms (Orth10, Orth11, and CH25) induced a modest increase in BDD-FVIII plasma levels (3-4-fold) compared with the wild-type FVIII-BDD construct (Orth40).
- Manually edited sequences such as those of Orth01, Orth04, and Orth23 induced much higher BDD-FVIII efficacy.

Construct	Codon optimization algorithm	Average FVIII expression at day 14 [IU/ml]	Number of mice	Fold increase vs wt
vOrth40	Human wild type	0.03	12	-
vOrth01	In-house	0.55	22	18.3
vOrth04	**In-house**	**2.21**	**55**	**73.7**
vOrth08	Radcliffe et al., 2008 (4)	0.11	6	3.6
vOrth23	JCAT modified	0.91	5	30.3
vOrth10	Eurofins	0.09	7	3.0
vOrth11	IDT	0.08	8	2.7
vCH25	GeneArt	0.13	18	4.3

Table 1. Expression of FVIII in plasma of FVIII knock-out mice induced by different AAV vector constructs.

- Based on these results, the Orth04 construct was selected as the BAX 888 lead candidate for further development (**Figure 2**).
- Interestingly, the plasmid encoding Orth04 also led to more efficient virion packaging and virus production.

Figure 2: Gene therapy vector AAV8-BDD-FVIIIopt

Structural analysis of vectors

The integrity of the vector genomes was analyzed using AAV agarose gel electrophoresis. The results shown in **Figure 3** demonstrate size-matching genomes of vOrth01, vOrth04, and vOrth40, indicated by a distinct DNA band in the corresponding lanes. Despite a vector size of approx. 5.2 kb, the genome is a homogenous band confirming correct packaging of the slightly oversized genome (relative to an AAV wild-type genome of 4.7 kb).

To confirm the expected pattern of capsid proteins of all vectors, SDS PAGE followed by silver staining was performed for vOrth01, vOrth04, and vOrth40 (**Figure 4**). The downstream purification procedure resulted in highly purified material displaying the expected protein pattern of VP1, VP2, and VP3. Each lane contained 1x10E+10 vg of the respective viral construct.

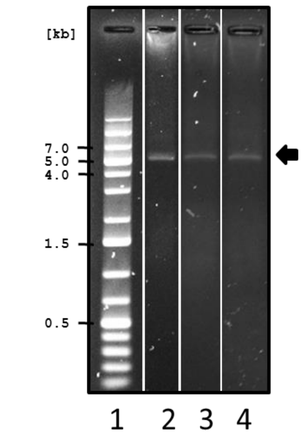

Figure 3. Integrity of AAV vector genome preparations. The AAV vector genomes migrate at approximately 5 kb (arrow, right side). Lane 1, DNA marker; lane 2, vOrth40; lane 3, vOrth01; lane 4, vOrth04.

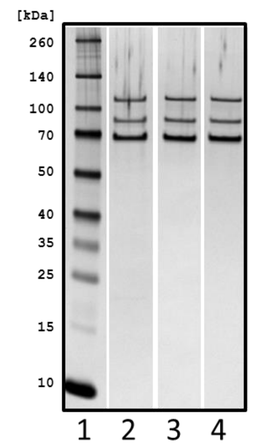

Figure 4 Protein analysis of selected AAV vectors. The vectors show matching AAV8 capsid patterns consisting of VP1, VP2 and VP3. Lane 1, Size marker; lane 2, vOrth40; lane 3, vOrth01; lane 4, vOrth04.

CONCLUSIONS

- Orth 04's features make it a promising drug candidate for Shire's FVIII gene therapy program (BAX 888).
- With this AAV8.BDD-FVIII construct, we hope to sustainably transform the bleeding phenotype of patients with hemophilia from severe into mild to moderate by a single treatment.

REFERENCES

1. Grieger,J.C. et al. (2015). Production of Recombinant Adeno-associated Virus Vectors Using Suspension HEK293 Cells and Continuous Harvest of Vector from the Culture Media for GMP FIX and FLT1 Clinical Vector. Mol. Ther. 2016 Feb;24(2):287-97.
2. Aurnhammer,C., et al. (2012). Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences. Hum Gene Ther Methods, 23, 18-28.
3. Fagone,P., et al. (2012). Systemic errors in quantitative polymerase chain reaction titration of self-complementary adeno-associated viral vectors and improved alternative methods. Hum Gene Ther Methods, 23, 1-7.
4. Radcliffe PA, Sion CJ, Wilkes FJ, Custard EJ, Beard GL, Kingsman SM, Mitrophanous KA. 2008. Analysis of factor VIII mediated suppression of lentiviral vector titres. Gene Ther.15(4):289-97.

DISCLOSURES

F.G. Falkner, F. Horling, J. Lengler, M. Weiller, J. Mayrhofer, M. Turecek, W. Höllriegl, H. Rottensteiner, and F. Scheiflinger are employees of Baxalta Innovations GmbH, now part of Shire. P.E. Monahan is an employee of Shire, Cambridge, MA. The study was sponsored by Baxalta Innovations GmbH, now part of Shire.