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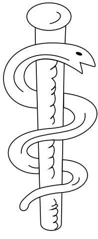

DIRECTED EVOLUTION OF AAV CAPSIDS FOR IMPROVED EFFICACY AND SPECIFICITY OF DELIVERY TO PRECLINICAL MODELS OF HUMAN LIVER

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Abstract:

Introduction and objective

Recombinant vectors derived from adeno-associated viruses (rAAVs) are the leading platform in human gene therapy applications, with high-profile examples targeting diseases of the central nervous system, eye and liver. The liver, quite likely a natural host organ for wild type AAV2, is a particularly attractive target for the development of AAV-mediated gene therapies. Despite large number of AAV variants current at various stages of development as carriers of liver therapeutics, thus far no liver-directed AAV-based therapy has obtained market authorization. Strong preclinical data is the cornerstone of any translational program, and while AAV bioengineering is commonly applied to try to develop novel human-tropic vectors for clinical applications, due to species-to-species differences, dedicated vectors to support preclinical work may need to be developed. Here we applied AAV directed evolution and *in vitro* selection to identify AAV capsids that target human liver cells *in vitro*.

Material and methods

Using DNA shuffling technology, we have generated a capsid gene library based on natural parental serotypes (AAV1 through AAV12). Shuffled capsid library was selected in a preclinical model of human liver.

Results

The AAV variants enriched based on their improved efficiency of transduction of a human hepatocyte cell line were vectorized and subsequently functionally characterized on human cell lines. This directed evolution method enabled us to select novel AAV variant, AAV-CH4.2. While the selected variant did not exceed the parental serotype in terms of transduction efficiency, it was substantially more efficient at packaging than its closest homolog, serotypes AAV6.

Conclusions

Based on its strong transduction profile and manufacturability, we believe that AAV-CH4.2 is a strong candidate for further evaluation and as a potential novel gene therapy vector for preclinical studies in human liver applications.

Keywords: gene therapy, AAV, adenovirus-associated viral vector, bioengineering.

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Introduction

Gene therapy brings a great hope for people affected by a broad spectrum of currently incurable inherited and acquired diseases. The basic concept behind gene therapy is the delivery of a functional gene into cells or tissues to remediate the consequences of pathology-causing mutations. Although number of technologies have been developed to facilitate delivery of genetic payload to target cells, *in vivo* delivery is limited to a handful of technologies, with methods based on the use of viral vectors gaining in popularity due to promising results in clinical trials. Particularly, adeno-associated virus (AAV) vectors are very promising, due to a lack of pathogenicity and low immunogenicity, wide range of cell tropism and long-term gene expression in non-dividing cells.

AAV is a nonpathogenic human parvovirus, first employed as a gene target vector as early as in 1984 [1]. AAV is a small, single-stranded DNA virus, which requires co-infection with a helper virus for efficient replication and viral gene expression. The AAV genome is protected on both ends by 145 base T-shape inverted terminal repeat (ITR) sequences. These structures are implicated in replication, second-strand synthesis, encapsidation, and insertion of the viral genome into an AAVS1 locus on human chromosome 19 [2]. The AAV genome encodes for four non-structural Rep proteins responsible for viral replication and three capsid proteins (VP1–3), in addition to an Assembly Activating Protein (AAP) encoded on an alternative reading frame within the *cap* gene [3]. Recently, new protein was discovered, called "Membrane Associated Accessory Protein" (MAAP), encoded on an alternative ORF in the VP1 region [4].

AAV-derived recombinant vectors (rAAV) represent one of the most powerful gene therapy tools and are currently under investigation in numerous clinical trials (for example phase III studies for hemophilia [5], phase I/II studies for Pompe disease [6] and Parkinson's disease [7]. Three gene therapy products based on natural AAV serotypes have reached the market. Those include Glybera (AAV1) for treatment of inherited metabolic disorder lipoprotein lipase deficiency (LPLD), unfortunately withdrawn from the market in 2017 [8], Luxturna (AAV2) for treatment of an inherited retinal diseases (IRDs) [9] and, most recently, Zolgensma (AAV9) for treatment of spinal muscular atrophy type 1 (SMA1) [10]. However, the high cost of large-scale production of clinical grade rAAV, as well as low efficiency of transduction of primary human cells, remain the main obstacles for clinical applications of AAV vector.

Since the AAV capsid is the primary determinant of vector tropism, as well as influences vector packaging efficiency (= yield), AAV capsid bioengineering has the potential to overcome the main limitations of the current AAV vectors. Specifically, AAV bioengineering has been shown to help overcome the limited tissue tropism, including cell binding, entry, endosomal escape, and trafficking, as well as vector targeting by the immune system [11, 12]. Many different approaches have been developed to engineer AAVs for the transduction of clinically relevant cells and organs [13-16]. Those have been extensively reviewed in Colella et al. [3], [17] and Wang et al. [18]. Capsid diversification techniques can be focus on the entire capsid or can be limited to specific regions, such as the hypervariable regions [19] located within the surface exposed loops. The most frequently utilized capsid modification strategies simulate the processes of natural evolution to create large and highly variable libraries of modified capsid variants with new properties. The capsid libraries can be generated through a number of molecular biology techniques, such as the introduction of point mutations in the capsid gene by error-prone PCR [20], the incorporation of random peptide libraries on the vector surface (AAV display peptide libraries) [21] as well as DNA shuffling. The latter technology is based on the recombination of parental genes, which are first fragmented and subsequently randomly reassembled, resulting in libraries of chimeric genes [12].

Following the generation of a sufficiently diverse pool of AAVs, the directed evolution is applied to facilitate screen for novel variants with desirable properties. Through submitting the heterologous library of AAVs to an experimentally controlled selective pressure, either *in vitro*, *in vivo* or *ex vivo*, this technique has been successfully applied to engineer novel AAV variants, such as for example variants capable of more efficient transduction of human pluripotent stem cells (hPSCs), human liver cells, murine and primate retina reviewed in Zinn [22].

The liver in particular is a key target for the development of more efficient AAV vectors, given its involvement in over 500 functions, such as metabolic activities. There are over 100 different liver diseases, both genetic and acquired, which reduce quality of life and significantly contribute to mortality, resulting in more than 2 million deaths per annum [23]. They also account for an unimaginable healthcare cost worldwide. Several natural serotypes of AAV have been developed as gene therapy vectors, with activities serotypes AAV2, AAV5, AAV8 and AAV9 among the most common choices for liver targeting [24]. Prototypical AAV2 has been the first AAV to be used in liver-targeted clinical study [25], although the clinical benefit was short lasting and weaker than would have been anticipated based on preclinical studies. UniQure carried out a clinical trial delivering the factor IX in adults with hemophilia B using AAV5 capsid (AMT-060) [26]. BioMarin Pharmaceutical used AAV5 to deliver the codon-optimized B domain-deleted F8 cDNA (BDD-FVIII) (BMN270) into patients with severe hemophilia A in phase 1/2 clinical trial, and subsequently expanded the testing of BMN270 into two phase 3 trials (NCT03370913, NCT03392974) [27]. Spark Therapeutics used a modified AAV8 capsid (SPK-9001) to deliver hyperactive FIX variant (R338L) Padua in a clinical trial (NCT03587116) for hemophilia B [28]. AAV8 has been evaluated in multiple liver-targeted clinical studies, with the most prominent being work performed by Nathwani et al towards the development of AAV-based gene therapy for hemophilia [29]. AAV9 was employed as a vehicle for administration of the SMN gene, the causative gene in SMA, in a clinical trial conducted by AveXis in SMA patients. The trial demonstrated remarkable improvements in motor milestones and rates of survival in the patients [30].

In addition to the natural variants, first wave of bioengineered variants has now reached the clinic. Spark Thera-

peutics used an engineered capsid AAV-LK03 to deliver BDD-FVIII (SPK-8011) in phase 1/2 clinical trial [31]. LK03 is the first clinically-tested AAV capsid produced by capsid shuffling and library selection, and it was shown to transduce human hepatocytes 100-fold better than AAV8 *in vitro*, along with being more resistant to NAbs than AAV2 [32]. Spark100 is another engineered capsid currently being prepared for phase 3 clinical trials, after demonstrating sustained therapeutic expression of FIX coagulant activity following gene transfer to Haemophilia B patients [33].

However, clinical development would not be possible without strong support of preclinical data often obtained from a number of various preclinical model systems. While later stages of preclinical development often utilize *in vivo* models, such as non-human primates, early stage and exploratory studies, such as those developing new therapeutic strategies for hundreds of genetic disorders affecting the liver, often rely on efficient delivery to more robust *in vitro* models, such as those based on immortalized hepatocytes.

Here we present outcomes of an AAV bioengineering approach used to develop novel AAV variants to support preclinical studies in the area of human liver gene therapy. The study involved creation of a diverse AAV capsid library, based on twelve natural parental serotypes, and a subsequent selection in a preclinical model of human liver. The strategy was designed to facilitate the accumulation of modifications within the AAV capsid that improve efficiency of transduction of immortalized human liver cells. In addition to giving rise to powerful vector candidates, these studies serve as a proof-of-concept for AAV development using more advanced preclinical models of human liver and other tissues.

Materials and Methods

Generation of shuffled capsid library

Shuffled AAV libraries were generated as previously described [12], with some modifications. The capsid genes from wt AAV serotypes 1 through 12 were cloned into plasmid based on the pGEM-T Easy System (Promega, cat. no A1360) and served as templates for PCR. Genes coding for capsids were amplified in standard PCR reaction, mixes at 1:1 molar ratio, and digested with 0.1 U DNaseI (NEB, cat. no. M030L) for 2-5 min. The pool of fragments was separated on a 1% agarose gel and fragments from 200 to 1000 bp were excited from the gel using ZymoClean Gel DNA Recovery Kit (Zymogen, cat. no. D4001T).

For primer-less PCR reassembly reaction, 500 ng of gel-extracted fragments was used. Fully reassembled capsids were amplified in a second PCR with primers F: GTACCAGTTCAGTCCAGTTGCA and R: CATGTGGATCCTGGTACGTGT carrying overlapping ends to pLL012_pGEM library rescue vector. A Gibson Assembly reaction was performed by mixing an equal volume of 2 × GA Master Mix (NEB, cat. no. E2611L) with 1 pmol PCR-amplified and DpnI (NEB, cat. no. R0176L) – treated pLL012_pGEM (Forward: 5'-ACTTGTTCACTTTGATGGCGAGG, Reverse: 5'-CTGCACACGACATGACATCACG) and 1 pmol of the recovered shuffled capsids. DNA was ethanol precipitated and electroporated into SS320 electro-competent *E. coli* (Lucigen, cat. no. 60512-2). The total number of transformants was calculated by plating 10-fold serial dilutions of the electroporated bacteria. The pool of transformants was grown overnight in 500 ml of Luria-Bertani media supplemented with trimethoprim (10 mg/mL) (Sigma, cat. no. T7883-5G). Ten individual clones were picked, sequenced and analyzed using Sequence Origin Depiction (SOD) plugin (https://github.com/CMRI-TVG/AAVcodons) created for Geneious (https://www.geneious.com/) [34] and Xover tool (http://qpmf.rx.umaryland.edu/xover.html).

Libraries were subsequently digested overnight with SwaI (NEB, cat. no. R0604L) and NsiI (cat. no. R0127L), and 1 mg of insert was ligated using T4 DNA ligase (NEB, cat. no. M0202) at 1:1 molar ratio into a replication-competent AAV2-based plasmid (pWK5), containing ITR2 and rep2, and unique SwaI and NsiI sites downstream of rep2. Ligation reactions were concentrated using ethanol precipitation, electroporated into SS320 electrocompetent bacteria cells, and grown as described above. Total pWK5 library plasmids were purified with an EndoFreeMaxi prep Kit (QIAGEN, cat. no. 12362).

AAV production and purification

All AAV vectors stocks were prepared by polyethylenimine (PEI) (Polysciences, cat. no. 239662) triple transfection (2:1 PEI:DNA ratio) of adherent HEK293T cells (ATCC, cat. no. CRL-3126). The pAd5 helper plasmid, AAV transfer vector expressing GFP, and an AAV-helper plasmid encoding rep2 and the capsid of interest were transfected at 2:1:1 molar ratio. For AAV capsid libraries (replication competent) only two plasmids were used: the pAd5 helper plasmid and the pWK5 libraries containing ITR-rep2-CapLibrary-ITR at a 1:1 molar ratio.

Cells were seeded 18 hr prior to transfection into five 15-cm tissue culture dishes to obtain 90% confluency at the time of transfection. Cells were harvested 72 hr post transfection and centrifuged for 15 min at 5,000 g. Media was mixed with ¼ volume of 40% PEG (Sigma-Aldrich, cat. no. 89510-1KG-F) in 2.5 M NaCl (Sigma-Aldrich, cat. no. 746398-500G) and incubated on ice overnight. After centrifugation at 5,000 g for 30 min at 4°C, PEG pellet was resuspended in 1 ml PBS buffer (pH 8.0). The cell pellet was resuspended in 2 mL of PBS buffer (pH 8.5) and subjected to three freeze-thaw cycles. Resuspended PEG pellet solution and the cell lysate were combined. Genomic and free plasmid DNA were removed by incubating with 200 U/mL of Benzonase (EMD Millipore, cat. no. 1016970001) at 37°C for 1 hr. Subsequently 10% sodium deoxycholate (Sigma-Aldrich, cat. no. D6750-25G) and 5M NaCl were added to a final concentration of 0.5% and 1M, respectively. Following incubation for 30 min at 37°C, cellular debris was removed by centrifugation for 30 min at 5,000 g.

Vectors were subsequently purified using iodixanol density gradients as previously described [35]. Amicon Ultra-4 Centrifuge Filter Units with Ultracel-100 kDa membrane (EMD Millipore, cat. no. UFC810024) were used to perform a buffer exchange and concentration step. Purified

AAV particles were stored in PBS buffer supplemented with 50 mM NaCl, 0.001% Pluronic F68 [v/v] (LifeTech, cat. no. 24040-032) at -80°C.

AAV titration

Vector preparations were titrated by real-time qPCR as described previously [36] using the following primers: GFP-F: 5'-TCAAGATCCGCCACAACATC and GFP-R: 5'-TTCTCGTTGGGGTCTTTGCT for vectors encoding the GFP, and rep2-F: 5'-AAGGATCACGTGGTTGAGGT and rep2-R: 5'-CCCACGTGACGAGAACATTT for replication-competent library preparations.

Library selection and vectorization of evolved AAV capsids

Human hepatoma HuH-7 cells (kindly provided by Dr Jerome Laurence, The University of Sydney) were seeded in complete media (DMEM with 10% [v/v] FBS) at 2 × 10^5 cells per well in 24-well dishes 18hrs prior to infection with AAV library. Six 10-fold dilutions of the AAV library were added to the media in duplicates. Cells were washed with PBS buffer 6 hrs after infection and wild type human adenovirus 5 (hAd5) (ATCC, cat. no. VR-1516) was added to facilitate AAV library replication. The plate without hAd5 served as a qPCR control. Cells were harvested 72 hrs after hAd5 infection and lysed by three freeze-thaw cycles. After each round of selection, AAV amplification was verified in each well by qPCR. To minimize cross-packaging of multiple vectors in single cell, the highest library dilution that resulted in no less than a 2-log increase in AAV signal, when compared to control well without wtAd5, was selected to the next round. Between selection rounds, heat inactivation (65°C for 30min) was used to inactivate wtAd5 in the samples. After the last, fourth round of selection, AAV capsid sequences were recovered from the wells by PCR using primers flanking the capsid region (Cap-F: 5'-CGATCTGGTCAATGTGGATTTGGATGACTGC, Cap-R: 5' GTAGTTAATGATTAACCCGCCATGCTACTTATCTACATGCAT). PCR-amplified cap genes were cloned into unique SwaI and NsiI sites of recipient helper packaging plasmid (pR2C) (downstream of the *rep2* gene), by digestion of the libraries overnight with SwaI and NsiI, and ligation of insert and plasmid with T4 DNA ligase at 3:1 molar ratios (1.3 mg of insert and 1 mg of plasmid). Ligation reactions were concentrated using ethanol precipitation, electroporated into SS320 electrocompetent bacteria, and grown as described above. Twenty individual clones were sequenced (Genomed S.A., Warsaw, Poland) to confirm progress of the selection process and to learn the sequence identity of selected capsids.

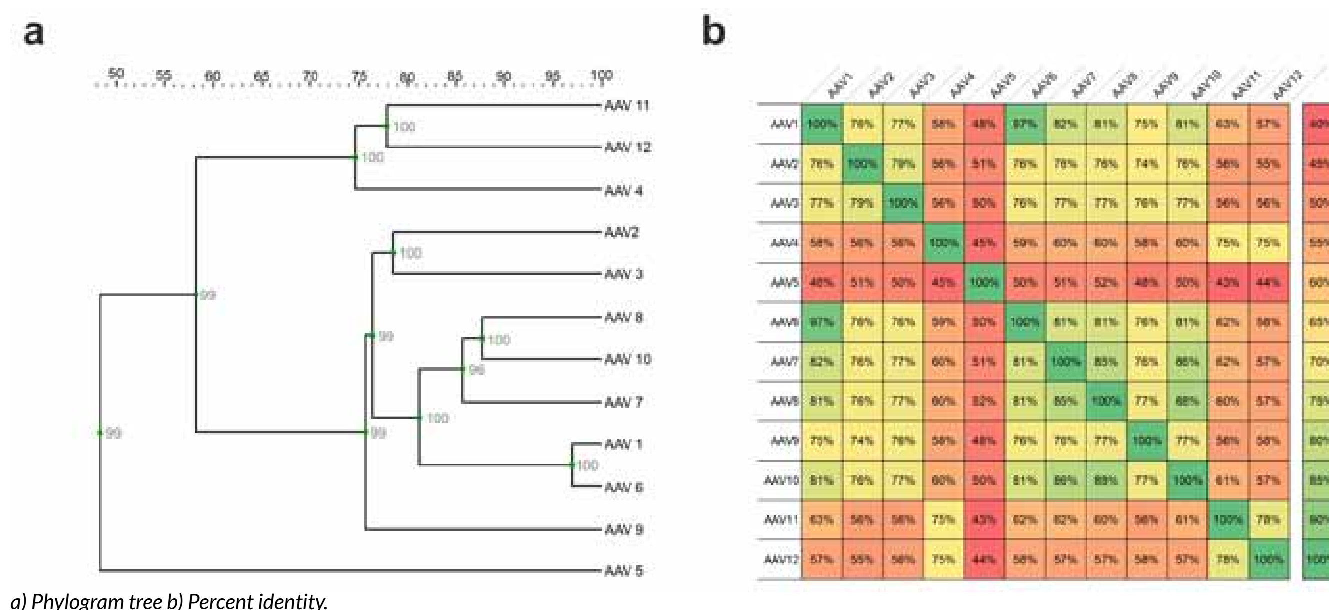
***In vitro* transduction analysis**

For transduction, HuH-7, HEK293 and A549 (ECACC, cat. no. 86012804) cells were plated at 2 × 10^5 cells per well into 24-well plates in complete media. Four hours later, the vector stock was diluted into 0.5 ml of complete media and added to the cells to achieve multiplicities of transduction (MOTs) of 10^5, 10^4, 10^3. The cells were harvested 72 hrs after transduction using TrypLE Express (Thermo Fisher, cat. no. 12604021), washed three times with PBS buffer and resuspended in 200 μl of cytometer buffer. EGFP expression was quantified in 20,000 cells using a BD FACS Calibur flow cytometer (Becton Dickinson, San José, CA, USA) equipped with a 488-nm argon-ion laser. Analysis of data was performed using Cell Quest Software (Becton Dickinson, San José, CA, USA). Results was shown as a percentage of cells with GFP fluorescence and mean fluorescence intensity (MFI) calculated for each sample (Department of Pathological Anatomy, The National Veterinary Research Institute, Pulawy, Poland).

Phylogenetic analysis of AAV sequences

Phylogenetic analysis of AAV sequences was performed using BioNumerics 7.6.3 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrogram was computed using multiple alignment with correction Jukes & Cantor and

Figure 1. Parental capsid phylogenetic relationship among 12 AAV variants used in the study.



a) Phylogram tree b) Percent identity.

clustering UPGMA method. Created phylogenetic tree was verified by cophenetic correlation coefficient (green dots).

Results

An AAV capsid library based on DNA shuffling of 12 different AAV parental serotypes was generated to enable selection of novel capsid variants on liver cells *in vitro*. The percent identity between the twelve capsid genes varied from 43 to 97 %, with cap 5 being the most distant serotype included in the mix (Fig. 1).

Specifically, DNA shuffling of capsid genes was performed by enzymatic fragmentation of parental AAV serotypes, followed by assembly of a full-length genes to create a diverse library of chimeras (Fig. 2).

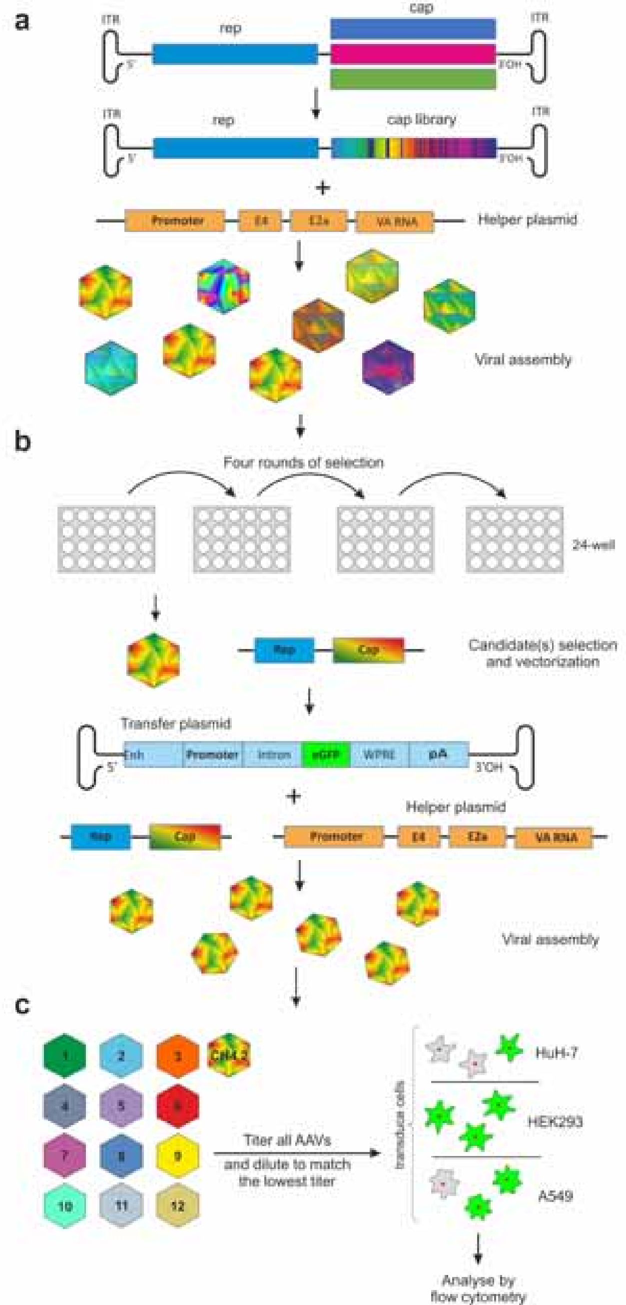
Library was cloned into a rescue vector and the capsid region of ten individual clones from the final plasmid library was Sanger sequenced. Analysis of the capsid gene sequences revealed that all clones were unique and highly diverse, containing fragments from multiple parental donor serotypes. Subsequently, the shuffled library was cloned into a replication-competent recipient construct (Fig. 2a) and additional ten clones were fully sequenced to confirm high quality of the final library prior to vector packaging step (Fig. 3a).

Detailed sequence analysis of the individual clones revealed that an average length of individual fragments from parental donors was 111.1 bp, average number of parental segments per clone was 19.3 ± 3.4 and average number of parental variants contributing to each chimera sequence was 8.5 ± 1.2. Shuffling index (percentage of individual clones containing at least one fragment >15bp from a given parental donor) was calculated and the results showed that the contribution of parental sequences from AAV6 and AAV1 was 32 % and 29 %, respectively. The remaining serotypes with considerable contribution were AAV7, AAV8 and AAV10 (shuffling index of 9.8 %, 9.8 % and 6.3 %, respectively). AAV5, AAV11, AAV12 and AAV4 contribution per single chimera was 1%, 1.1%, 0.3%, and 0.2%, respectively (Fig. 3b).

The results confirmed extensive genetic diversity and the presence of all twelve parental viruses in the final library pool with a bias toward AAV6 and AAV1 serotypes.

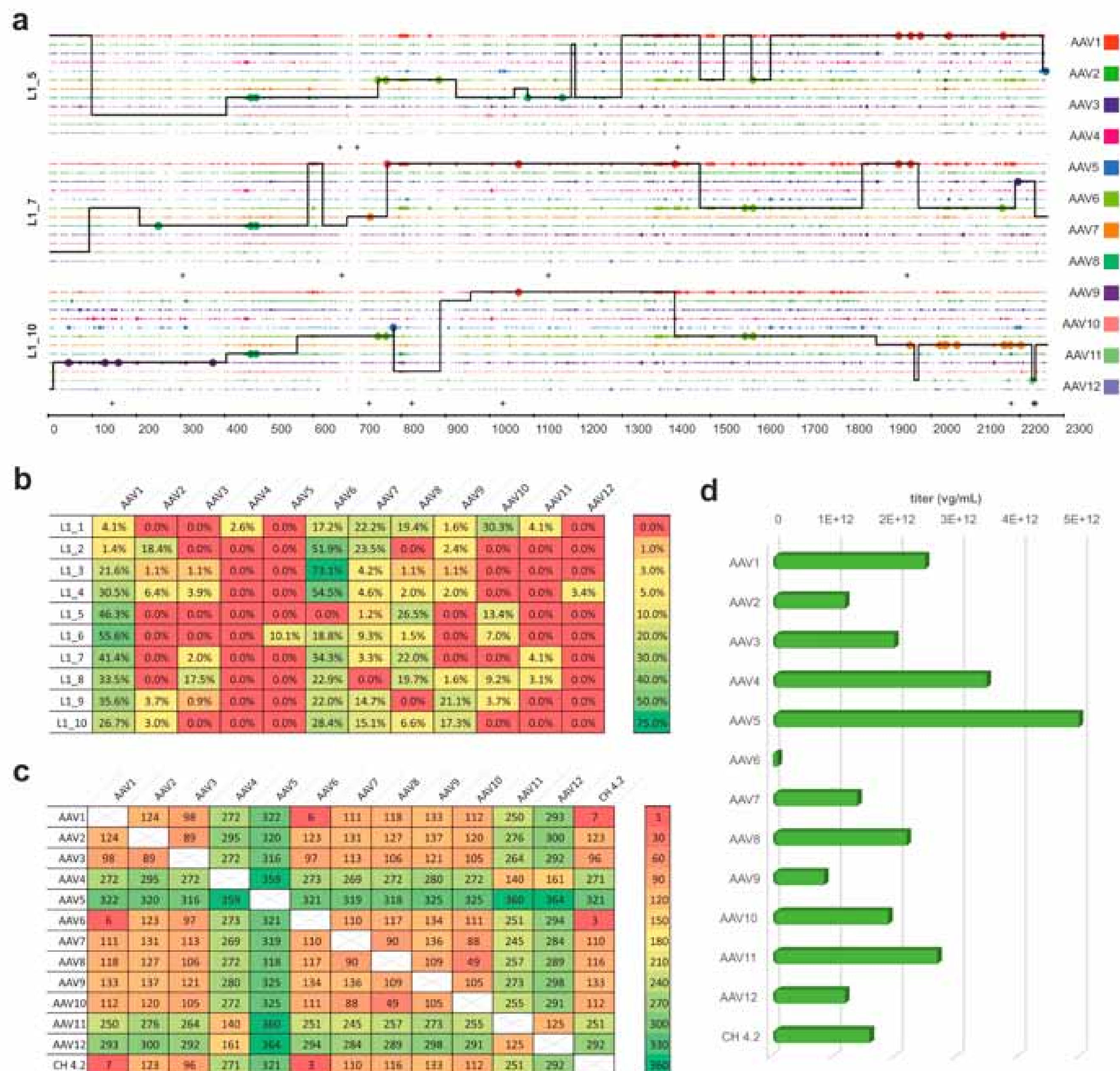
To screen for capsids with enhanced efficiency in liver, the library was packed resulting a particle titer ~2.9×10^{13} genomes/ml in the total volume of 500 µl. Produced AAV library was used to perform four rounds of selection on hepatoma cells (HuH-7) in the presence of wild type human adenovirus type 5 (Ad5) to support AAV replication. Total 4 round of iterative selection were performed and after the last round of selection, 17 clones were selected and sequenced to verify completion of the selection process. Sequencing revealed successful enrichment of a single chimera (AAV-CH4.2), which represented over 90 % of randomly selected clones. Sequence of AAV-CH4.2 revealed predominant homology to serotypes AAV6 and AAV1. On the nucleotide level, the capsid gene coding for AAV-CH4.2 was 98.55 % similar to AAV6 and 97.42 % AAV1. On the amino acid level, the sequence identity was

Figure 2. Schematic representation of the directed evolution of AAV capsids by DNA shuffling and functional validation of selected novel variants.



a) AAV capsid (cap) genes from parental AAV1 to AAV12 were digested with DNase followed by primer-less PCR to reassemble full-length shuffled genes. The shuffled capsid genes were inserted into a plasmid carrying AAV2 ITRs and AAV2 rep gene. Resulting plasmid library was transiently transfected together with an adenoviral helper plasmid (pAd5, encoding E4, E2a and VA adenoviral genes required for AAV replication), into HEK293 to produce viral AAV library.
b) AAV library selection was performed by coinfecting the cultured liver cells with the library and wild-type adenovirus type 5 (wtAd5), which enabled replication of AAV variants that were able to effectively infect the target cells. Capsid gene from the selected AAV variant was subsequently PCR amplified and cloned into an AAV plasmid carrying the AAV2 rep gene to generate AAV packaging construct. The AAV packaging plasmid was then transfected together with pAd5 and the transfer plasmid (plasmid encoding a standard expression cassette consist of the green fluorescent protein (GFP) transgene, polyadenylation signal (pA), and woodchuck post-transcriptional regulatory element (WPRE)) into HEK293 cells to produce final AAV-GFP for functional studies.
c) The new vector was used to transduce target cells and GFP expression was quantified. Parental AAVs were included as references.

Figure 3. Analysis of the capsid gene sequences and vector yield comparison.

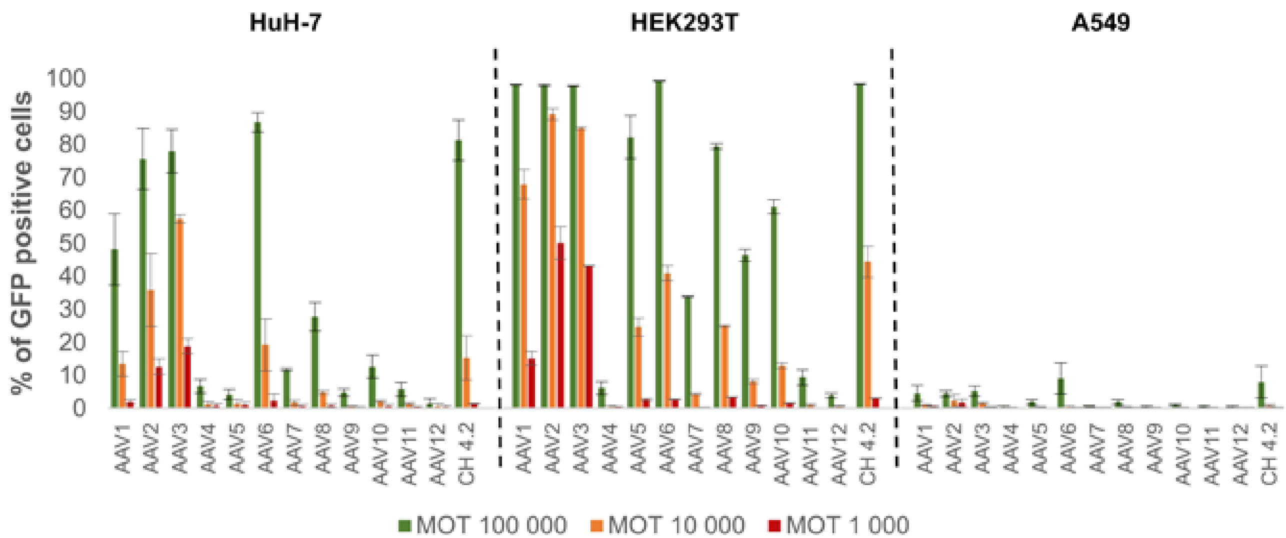


a) Amino acid composition analysis of shuffled capsid clones in the final plasmid library. Parental AAVs are shown in different colours. Size of the dot represents probability level that given sequence originated from that particular parental serotype. The solid black line for each chimera (L1_5, L1_7, L1_10) outlines the parental composition of given variant. A plus sign "+" indicates a de novo mutation that cannot be tracked back to any of the parental donors.
b) Shuffling index, percentage of individual clones containing at least one fragment <15bp from a given parental donor, between the capsid library, based on sequence composition of the 10 random clones (L1_1-L1_10), and the parental serotypes, with green color indicating higher and red indicating lower shuffling index.
c) Amino acid identity score (number of differences at the amino acid level) between individual parental serotypes (AAV1-12) and generated chimera CH4.2, with red color indicating higher and green indicating lower identity. D) Vector yield comparison of the novel selected chimera and parental variants.

99.59 % and 99.05 % to AAV6 and AAV1, respectively, or in other words, the difference between them was 3 and 7 amino acids, respectively (Fig. 3c).

Next, the transduction efficiency of the selected variant was assessed on human cell lines of various hepatocytes origin, namely hepato-carcinoma 7 cells (HuH-7), as well as non-liver cell lines, namely embryonic kidney cells (HEK293) and lung carcinoma cells (A549). To do so, the cap AAV-CH4.2 gene coding sequence was recovered by PCR and cloned into a standard helper plasmid downstream of the AAV *rep2* gene. This AAV-CH4.2 packaging plasmid was subsequently used to package AAV vectors encoding GFP reporter protein under the control of a ubiquitous CAG promoter. The same reporter cassette was also packaged into the twelve parental variants (AAV1-12), which were to be used as reference controls in the transduction study. However, this also allowed for direct vector yield comparison. All vectors were titrated by qPCR, and the titre from iodixanol production ranged from 5.3×10^{12} (AAV5) to 1×10^{11} vg/ml (AAV6) (Fig. 3d). Although at the sequence level our novel variant AAV-

Figure 4. Results of flow cytometry analysis for each cell type, individually transduced with parental AAVs and new vector variant (AAV-CH4.2).



-CH4.2 was very similar to AAV6, AAV-CH4.2 packaged substantially more efficiently than AAV6 with the final titer 10× higher than AAV6. All vectors were diluted to the same concentration and were used to transduce the above-mentioned cell lines at 3 different multiplicities of transduction (MOTs, number of vector particles per target cell): 100×10^3, 10×10^3 and 1×10^3. The cells were harvested 72 hours later, and the efficiency of transduction was analyzed by flow cytometry based on the level of GFP protein expression (Fig. 4).

As shown in Figure 4, the levels of transduction differed substantially between cell lines for all AAV tested. Not surprisingly, higher MOT led to an overall higher level of transduction in all groups. On the HuH-7 cells, AAV2, AAV3, AAV6 and AAV-CH4.2 outperformed all the other vectors, and at MOT 100×10^3 functionally transuded 75.7 %, 78.1 %, 86.7 % and 81.4 % of HuH-7 cells, respectively. Remaining vectors transduced HuH-7 cells less efficient and could be grouped into average transducers (AAV1 48.3 %, AAV8 27.8%) and poor transducers (AAV10 12.6 %, AAV7 11.7 %, AAV4 6.7 %, AAV11 5.8 %, AA9 4.9 %, AAV5 4.1 % and AAV12 1.5 %). The level of transduction of AAV-CH4.2 was comparable with the best vector (AAV6) and more than ten times better than AAV4, AAV11, AAV9, AAV5 or AAV12.

On the HEK293 embryonic kidney cells, AAV1, AAV2, AAV3, AAV6 and AAV-CH4.2 vectors reached a saturation levels of transduction (98.3 %, 98 %, 97.8 %, 99.4 %, and 98.5 % GFP positive cells, respectively) at MOT 100×10^3. At MOT 10×10^3, AAV1, AAV2, AAV3, AAV6 and AAV-CH4.2 transduced 71.1%, 90.4%, 85.3%, 42.7% and 47.8% cells, respectively. AAV5, AAV8 and AAV10 constituted an average transducer group (26.5%, 25.2%, 12.3%, respectively), while AAV9, AAV7, AAV11, AAV12, and AAV4 were the poorest transducers (7.7%, 4.6%, 1.3%, 0.9%, and 0.8%, respectively). Even though the transduction level of AAV-CH4.2 was lower than AAV1, AAV2 and AAV3, it was still more than forty times better than AAV11, AAV12 and AAV4.

The lung carcinoma line, A549, was the most resistant to AAV transduction, with AAV6 and AAV-CH4.2 leading to the highest level of transduction of 9.1 % and 8.1 %, respectively. AAV1, AAV2 and AAV3 transduced 4.6 %, 4.5 % and 5.3 % of the cells, respectively, while the remaining vectors transduced only 1% or less of the A549 cells.

Discussion

Currently, most AAV-based vectors used in gene therapy are derived from naturally occurring serotypes (e.g. AAV2, AAV5, AAV8). Their ability to transduce target tissues in clinical trials often turns out to be much lower than anticipated based on data from preclinical studies, which is why more functional variants for clinical applications are still in high demand. Strategies that enable shuffling AAV capsids from closely related (e.g. AAV1, 2, 3, 6) as well as evolutionarily distant (e.g. AAV5) serotypes enable generation of large pools of random variants (AAV libraries), each with a unique sequence and function. Such large libraries form a starting point in directed evolution strategies that enable selection for new variants with desired properties using preclinical models of human organs. The selection pressure applied can direct the selection process (hence Directed Evolution) towards identification of variants with new critical properties, such as increased transduction efficiency or specificity towards target cells.

However, as the novel bioengineered variants become more efficient at transduction of primary human cells *in vivo*, they often become less efficient at transgene delivery in preclinical models, such as immortalized cells. In fact, recent data suggests that some capsid properties that help with efficient transduction *in vitro* may differ from those required for *in vivo* function. For example, strong binding to heparan sulfate proteoglycans (HSPG), which help with transduction of cells in tissue culture settings make the vectors inefficient at tissue entry and transduction *in vivo* [37]. This suggests that as the field moved towards more efficient AAV variants for clinical

applications, parallel efforts may be required to develop tools in support of early preclinical work.

To this end, in the present study, the *cap* gene shuffling of 12 AAV serotypes was performed, followed by selection on human liver cell line, resulting in the identification of a novel variant (AAV-CH4.2). Functional analysis using three-cell lines revealed that AAV-CH4.2 was one of the most efficiently transducing variants when compared to parental AAVs. The *cap* AAV-CH4.2 sequence at the amino acid level is very similar to the *cap* sequence of AAV6 and AAV1, which could be related to the fact that those two serotypes were the most represented ones in the shuffled library used in this study. The overrepresentation of AAV1 and AAV6 may be due to the fact, that high identity of the *cap* DNA sequences directly influences the efficiency of shuffling of the input parental sequences, leading to significant advantage of sequences contributed by the closely related AAV serotypes [34]. AAV1 and AAV6 have the highest pairwise identity among the parental AAVs (97.1%), and together with AAV7, AAV8 and AAV9 belong to the same clade. More distant parental serotypes, such as AAV5, AAV4, AAV11, and AAV12, did not shuffle efficiently with other variants and were somewhat under-represented in our initial library. Results obtained in our study coincide with the phenomenon described in the literature, that typically evolved capsids differ from a single parent by only up to seven residues (this depends on the library type but usually corresponding to less than 1% of the capsid protein) [12].

It is known that AAVs attach to specific receptors on the cell surface in a serotype dependent manner. The attachment of most of the AAV serotype vectors is first mediated by binding to various cell surface or extracellular matrix proteoglycans, which serve as primary receptors. To enter the cells, interactions with additional co-receptors that mediates endocytosis are required [38]. More recently, type-1 transmembrane protein was shown to be required for transduction by most of AAV serotypes and was named the AAV receptor (AAVR) [39]. To compare the transduction efficiency of the selected variant with parental serotypes, cell lines from different human tissues were transduced with AAV-CH4.2 encoding GFP reporter, using parental AAV1-12 as controls. Studies showed that the newly selected vector AAV-CH4.2, together with closely related AAV6, were among the best transducing vectors on each of the tested cell lines, which is a very promising result. The second most effective vectors (AAV2 and AAV3) belong to one clade, and the weakest transducers (AAV4, AAV11 and AAV12) also belong to one clade, a fact that can potentially explain similarity in transduction pattern. Different transduction levels on studied cell lines, especially very weak transduction on A549 cells, can point towards the existence of a potential barrier preventing AAVs from cell attachment on cell entry. As described in the literature, AAV receptor (AAVR) has specific and restricted expression pattern, for example it is weakly expressed on airway epithelial cells. For most AAVs, which rely on this receptor for cellular entry, this could impose a strong barrier that holds back the vectors from entry [40].

Currently gene therapies are among the most expensive therapeutics available, which limits the access for many patients and thus lowers the overall impact on the patient population. The high cost of gene therapeutics is in part driven by the high cost of vector manufacturing, which also creates a substantial roadblock for many promising translational programs. An efficient vector production is essential for successful commercial implementation of gene therapy. While preclinical studies require substantially lower amounts of vectors, efficient manufacturing is still an important factor when considering a choice of vector. Despite being highly similar to AAV6 at the amino acid sequence and function levels (Fig 4) the selected AAV-CH4.2 can be packaged at substantially higher yield (Fig 3d), increasing its overall value and utility.

The presented data illustrate the utility of AAV bioengineering for the development of AAV vectors for preclinical studies, while the vector packaging and functional data position AAV-CH4.2 as a strong candidate for further evaluation and as a starting point for further bioengineering applications.

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Declarations. Conflict of Interest

The authors declare no conflicts of interest.

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