Engineering AAV capsid variants to overcome pre-existing immunity and improve gene delivery to human liver

Poster M-3
Abstract 122

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Introduction

- Vectors based on adeno-associated virus (AAV) offer robust and long-term gene expression and are commonly used in gene therapies.
- However, continued challenges remain regarding immunogenicity and toxicity of AAV at high vector doses.
- Through concerted effort from the field, a small number of capsid variants demonstrating improved tropism for human liver cells have emerged, including our first-generation capsid, AAVS3.[1–3]
- This report focuses on the development and characterization of capsid variants arising from directed evolution on primary human hepatocytes in the presence of highly AAV-neutralizing human plasma.

Methods

Selection of capsid variants

- AAV parental capsid sequences were shuffled by fragmentation and re-assembly and the shuffled library was used to produce replication-competent AAV (rcAAV) followed by purification by iodixanol density gradient (diversity 7 × 10^5 variants).
- Selection was performed by directed evolution (DE) on primary human hepatocytes over five rounds with the inclusion of a strongly neutralizing plasma pool.
- PacBio® long-read sequencing was used to track diversity and identify unique variants for characterization (upper panel, **Figure 1**).

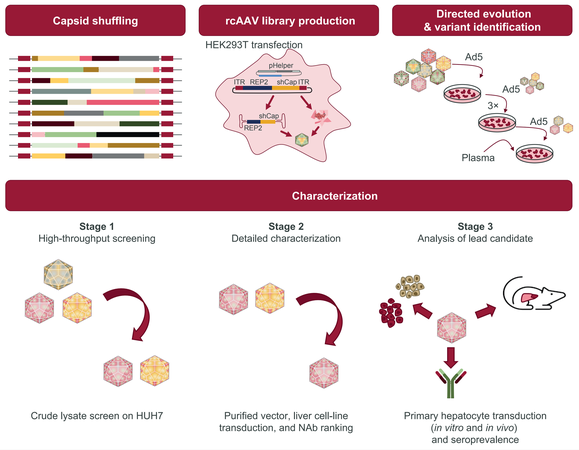
Screening and characterization of capsid variants

- In total, 26 candidates were selected for analysis in the characterization pipeline (lower panel, **Figure 1**).
- This consisted of a crude lysate screen on HUH7 cells (Stage 1); Transduction and antibody escape ranking (Stage 2); Evaluation of lead candidates in primary human hepatocytes (*in vitro* and *in vivo*) and a prevalence screen (Stage 3).

Antibody neutralization

- Assessments of neutralizing antibody (NAb) prevalence and comparisons of seroprevalence studies are complicated by:
  - the sample population size and demographics (age, ethnicity, geography, etc.)
  - assay specificity and sensitivity
- A highly sensitive, high-throughput, transduction inhibition assay (TIA) (**see Poster Tu-277**) was utilized in two formats during the characterization stages.
- Initially, antibody escape was ranked using the TIA to establish IC50 curves against an IVIG standard (**Figure 3B**).
- Subsequently, the increased sensitivity of our assay allowed stratification of the NAb response to capsid variants in a population of 96 healthy donor plasma samples (**Figure 5**), which allowed us to detect shifts in median titers and identify variants that expand the negative responder pool.

Figure 1: Selection and characterization of capsid variants

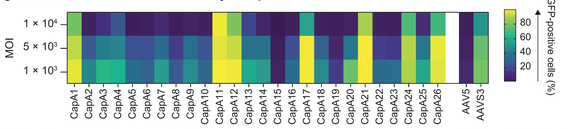


Results

Stage 1: High-throughput transduction screening

- Transduction of HUH7 cells revealed capsid variants with varying potency, some of which outperformed AAVS3 (**Figure 2**).
- Twelve variants representing a range of transduction efficiencies were selected for further analysis.

Figure 2: Variable transduction efficiency of capsid variants on HUH7 cells

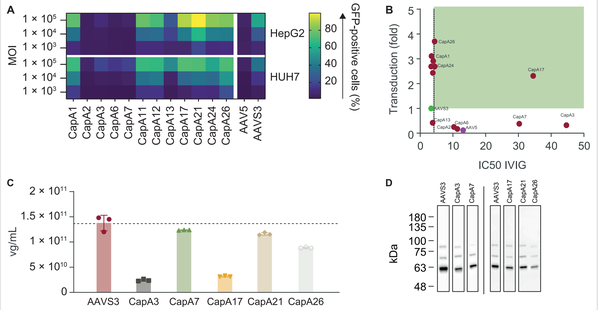


26 AAV candidates were produced at small-scale (6-well format) and transductions were performed at a range of MOIs on HUH7 cells using crude lysate after freeze-thaw. The transduction efficiency was determined as percentage GFP-positive cells by flow cytometry.

Stage 2: Detailed characterization

- In HUH7 and HepG2 cells, CapA17 and CapA21 displayed the highest transduction efficiency (**Figure 3A**).
- Capsid variants CapA3, CapA7, and CapA17 showed an improved antibody escape compared with AAVS3 and AAV5 (**Figure 3B**).
- A small subset of variants were assessed for manufacturability as determined by yield, and this revealed significant differences between variants (**Figure 3C**). All variants displayed VP1, 2, & 3 at expected ratios (**Figure 3D**).
- Capsid variant CapA17 was selected for further characterization.

Figure 3: Transduction efficiency, NAb ranking, and manufacturability of lead variants

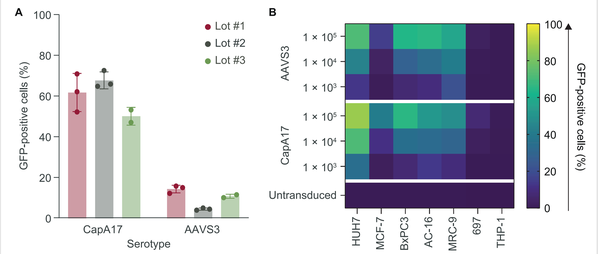


(A) Transduction efficiency of candidate AAVs, purified by iodixanol gradients, was assessed by flow cytometry for eGFP on HUH7 and HepG2 cells at a range of MOIs. (B) Fold difference to AAVS3 in HUH7 at an MOI of 1×10^4 was plotted against the IC50 from the antibody escape experiment using IVIG. (C) Manufacturability of a subset of capsid variants was tested in context of the split-packaging plasmid system in 293T cells seeded in 6 cm-dishes. (D) Analysis of Cap expression of crude cell lysates (samples shown in panel C) in Western blots probing with the B1 antibody. Lysates were loaded according to total protein concentration (3.0 µg per lane)

Stage 3: Analysis of lead candidate

- Transduction efficiency of CapA17 was greater than AAVS3 across three batches of primary human hepatocytes (**Figure 4A**).
- Comparison of transduction efficiency of CapA17 and AAVS3 in cell lines representative of key tissue targets revealed similar profiles (**Figure 4B**).

Figure 4: Lead variant shows improved transduction efficiency in vitro



(A) Primary human hepatocytes from 3 different donors were transduced at an MOI of 1×10^5 vg, and GFP-positive cells detected by flow cytometry. (B) Transduction efficiency in different cell lines was determined as percentage of GFP-positive cells to define specificity of CapA17 in comparison to AAVS3. Both vectors used a CMV promoter.

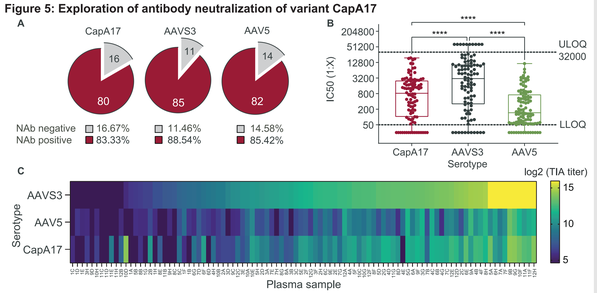
Exploring antibody neutralization of capsid variant CapA17

- Using the TIA on a 96-sample plasma bank, we observed differences in NAb prevalence and median titer.
  - CapA17 had the highest number of samples (16/96) that were non-reactive (no neutralization at any level) (**Figure 5A**).
  - Median NAb titer of CapA17 was greater than AAV5, but less than AAVS3 (**Figure 5B**).

CapA17 exhibits a distinct NAb TIA profile

- Direct comparison of NAb titer across capsids in individual samples provides insight into cross-reactivity.
- Using the TIA on a 96-sample plasma bank, we observed that not all samples neutralized transduction to similar levels across the three capsid variants (**Figure 5C**).
  - This head-to-head comparison highlights the power of directed evolution to generate divergent capsid variants with distinct neutralization profiles, and that the cross-reactivity of a given plasma sample is not absolute.

Figure 5: Exploration of antibody neutralization of variant CapA17



(A) A sample was classed as NAb positive if any level of neutralization was detected in the TIA assay for CapA17, AAVS3 and AAV5. (B) Box Whisker diagram of individual IC50 values for a plasma bank of 96 samples. A sample was classed as negative if it was below the LLOQ. The lines represent median TIA titer, and one-way ANOVA compared serotype mean TIA titers (**** $p < 0.0001$). Samples with values < 50 were assigned a value of 25; samples with values >32000 were assigned a value of 64000. (C) Comparison of the NAb TIA profiles based on the log2 (TIA titers).

Conclusions

- Utilizing capsid shuffling and directed evolution, we identified capsid variants presenting unique transduction and antibody escape properties.
- The capsid variant, CapA17, showed superior transduction properties in hepatic cell lines and primary human hepatocytes compared with AAVS3 and AAV5.
- Assessment of NAb prevalence in our TIA provided a rapid view of antibody escape and cross-reactivity of new variants in a relatively large sample size.
- CapA17 showed reduced NAb titers compared with AAVS3; however, its manufacturability requires optimization.
- This proof-of-concept study highlights the challenges of identifying variants with all desired characteristics. However, this work shows the power of such discovery efforts to better understand structure-function relationships.

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Abbreviations: 697, human B cell precursor leukemia; AAV, adeno-associated virus; AC-16, proliferating human cardiomyocyte cell line; Ad5, adenovirus type-5; ANOVA, analysis of variance; BxPC3, human pancreatic cancer cell line;GFP, green fluorescent protein; HEK293T, human embryonic kidney 293T-derived cell line; HepG2, human hepatocellular carcinoma-derived cell line; HUH7, human hepatocellular carcinoma-derived cell line; IC50, half-maximal inhibitory concentration; ITR, inverted terminal repeat; IVIG, intravenous immunoglobulin; LLOQ, lower limit of quantification; MCF-7, human epithelial breast cancer cell line (adenocarcinoma); MOI, multiplicity of infection; MRC-9, human embryonic normal lung fibroblast; NAb, neutralizing antibody; NGS, next-generation sequencing; rcAAV, replication-competent adeno-associated virus; THP-1, human leukemia monocytic cell line; TIA, transduction inhibition assay; shCap, shuffled capsids; ULOQ, upper limit of quantification; vg, vector genomes

References:
1. Dane A *et al. Hum Gene Ther.* 2017;28(11):A103.
2. Dane A *et al. Blood.* 2018;132(Suppl 1):2197.
3. Dane A *et al. Hum Gene Ther.* 2021;32(19–20):A108.

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