AAV DNA Shuffle Library of GH Loop Regions for Directed Evolution of Cardiotropic Capsids

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INTRODUCTION

Tenaya Therapeutics has established integrated internal capabilities to broadly enable modality agnostic target validation and the identification, selection and optimization of capsids and components best suited to constructing and manufacturing adeno-associated virus (AAV)-based genetic medicines intended to target the underlying cause of cardiac diseases.

AAV-mediated gene transfer targeting cardiomyocytes (CMs) is a promising approach for treating genetic cardiomyopathies. However, current approaches often necessitate high vector doses when administered systemically to ensure gene transfer nearing 100% in CMs, which can lead to adverse effects, including immune responses, off-target transduction or hepatotoxicity. Lowering the required vector dose has the potential to minimize the risk of adverse effects and make gene therapy more economically feasible and accessible to larger patient populations. Development of novel AAV capsids with improved tropism to CMs is critical to enable the next generation of therapeutically relevant cardiac gene expression at lower doses.

The five variable regions (VRs; VR-IV to -VIII), located between the beta sheets G and H in the AAV capsid protein VP1, are known to play a key role in the receptor binding and internalization of AAV (Fig 1). To expand our search for capsid variants targeting CMs, we developed a novel AAV capsid library, the GH loop Variable Regions (GHVR) shuffle library. By randomly shuffling the five VRs from the 16 natural AAV serotypes onto the AAV9 capsid backbone, we established a rational strategy for discovering new capsids (Fig 2). This strategy enables a focused exploration of the genetically diverse molecular space crucial for infectivity, aiding in the identification of novel capsids tailored for targeted gene delivery to CMs.

The GH loop consists of five variable regions crucial for various AAV functions and is located between the two beta sheets

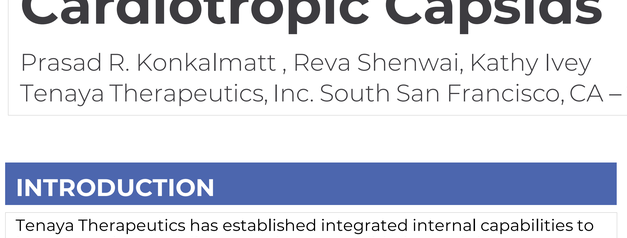


Figure 1. **A**) Diagrammatic representation of the positions of beta sheets (βA – βI, light grey boxes) and variable regions (VR-I – VR-IX, magenta boxes) across the AAV capsid protein VP1 (solid dark grey box). The green shade indicate the five variable regions nested between the two beta sheets G and H. **B**) The ribbon model showing the organization of the VP1 protein backbone depicting the locations of the beta sheets, variable regions, and the green shade indicate the five VRs within the GH loop. Major receptor binding domains, VR-IV and VR-VIII are shown in red and green respectively.

OBJECTIVE

Create novel capsids with improved tropism for CMs through a unique capsid library generated by shuffling the five variable regions within the GH loop of AAV serotypes. This innovative strategy exploits the intrinsic properties of these variable regions in the GH loop, which direct the tropism of AAV serotypes.

MATERIALS AND METHODS

AAV GHVR library preparation

The GHVR shuffle library was created by assembling the synthetic DNAs each carrying a VR sequence of 16 AAV serotypes flanked by the AAV9 conserved sequences. For each of the 16 AAV serotypes, 5 synthetic DNAs were created with the serotype specific VRs flanked by the AAV9 conserved sequences. The synthetic DNA were also designed to carry

MATERIALS AND METHODS, cont'd

overlapping sequences to enable random assembly of five sequential VR regions from 16 different serotypes by Gibson assembly. This strategy produced a theoretical capsid pool to a manageable size of 1.05E+6 capsid variants. The GHVR capsid library was packaged by triple transfection method in HEK-293 cells and purified by Iodixanol gradient centrifugation.

Screening of GHVR capsid library

AAV GHVR capsid library was screened sequentially in mice and cynomolgus monkeys. The capsid library was administered into mice (8-10 weeks, male, n=3) intravenously at the dose of 7.5E+12 gc/Kg body weight. Four weeks after, heart and liver tissues were collected from the mice, the GHVR regions of the capsid variant that successfully transduced the heart tissue were amplified by PCR. The PCR amplicon was inserted into the corresponding site in AAV9 VP1 to generate the second plasmid library of GHVR variants and then the second AAV library. The second AAV library was administered into cynomolgus monkeys (5-6 years, male, n=2) intravenously at the dose of 1E+13 gc/Kg body weight. Four weeks after, heart and liver tissues were collected from the NHPs, the GHVR regions of the capsid variant that successfully transduced the heart tissue were amplified by PCR.

Next Generation Sequencing and Analyses

The capsid variants from the animal hearts were identified through Next Generation Sequencing (NGS), employing 250 x 250 paired-end sequencing of the PCR amplicons spanning the variable regions IV to VIII. The R1 reads were combined with the reverse complement of their respective R2 reads to generate complete sequences. Any sequences shorter than 400 base pairs were excluded from further analysis. The frequencies of each unique sequence within the dataset were determined using a custom-built R programming script that was generated at Tenaya.

RESULTS

Generation of GH loop variable regions shuffle library

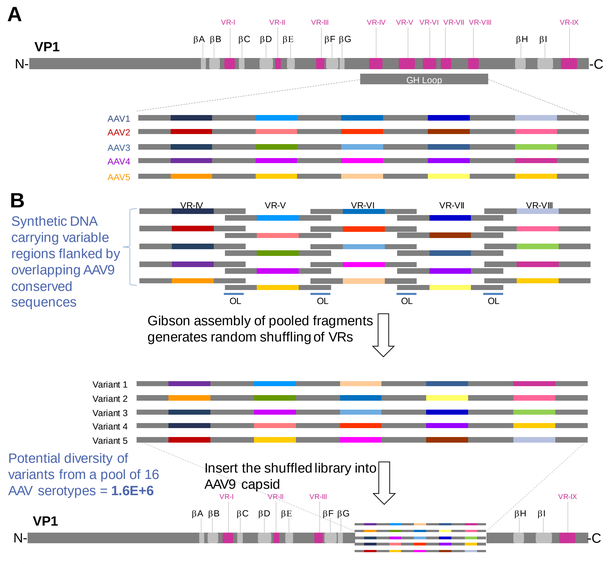


Figure 2. Showing the strategy for the generation of GH loop variable regions shuffle library. A) The five variable regions within the GH loop for each AAV serotype are indicated by the shades of, blue for AAV1, red for AAV2, green for AAV3, purple for AAV4, and orange for AAV5. B) Synthetic DNA fragments carrying a VR sequence for the various AAV serotypes flanked by overlapping sequences indicated by black line (OL), enabling random assembly of five sequential VR regions from 16 different serotypes by Gibson assembly to produce GHVR library of AAV capsids.

RESULTS, cont'd

Two-species Screening Strategy for GHVR Library

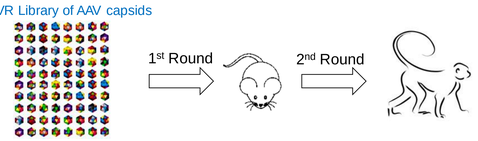


Figure 3. The AAV GHVR capsid library were screened sequentially in mice and cynomolgus monkeys. First, the library was administered to mice, and the capsid variants that transduced mouse hearts were amplified via PCR. Subsequently, the PCR amplicons were inserted into the AAV9 VP1, generating the second AAV library. This second library was then administered to cynomolgus monkeys. The capsid variants that transduced monkey hearts were amplified by PCR for NGS analyses.

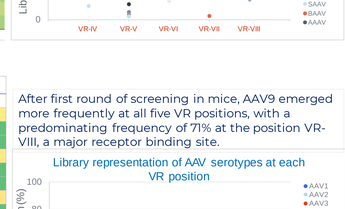
Occurrence and Enrichment of Variable Regions at Screening Stages

A

GHVR Library

	VR4	VR5	VR6	VR7	VR8
AAV1	14284	9323	11847	21900	19577
AAV2	21304	14850	5477	22961	21009
AAV3	16189	11117	6008	17214	15418
AAV4	16843	4899	22801	20716	21357
AAV5	25098	20356	5443	20982	25182
AAV6	13235	9971	19350	22371	24321
AAV7	22574	20129	17699	12510	17456
AAV8	25698	32516	7504	20987	25098
AAV9	18427	17189	14881	26468	26147
AAV10	15953	26509	26810	21333	23098
AAV11	16474	2376	21564	16362	21066
AAV12	13546	1646	22184	21568	21009
AAV13	24195	223448	6398	19422	9556
SAAV	5331	1081	7553	15688	7493
BAAV	22876	24109	14881	1365	10987
AAAV	17205	24654	6526	9823	8234
Total	289232	243069	216926	291670	297008

In the initial library, all 16 AAV serotypes are evenly represented across five positions, especially at VR-IV, -VII, and -VIII, near the expected ratio of 6.25%.

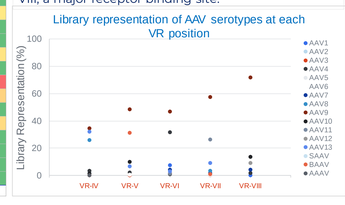


B

HT1-Library

	VR4	VR5	VR6	VR7	VR8
AAV1	0	0	11847	825	152
AAV2	3068	0	0	0	0
AAV3	0	402	2808	311	0
AAV4	5740	4108	48925	0	3884
AAV5	1683	1045	0	44	0
AAV6	0	1045	4279	825	0
AAV7	0	0	6365	3273	9347
AAV8	45698	2	0	5555	0
AAV9	60599	85798	72213	97012	164489
AAV10	2188	17467	1751	0	30985
AAV11	0	0	1904	44252	0
AAV12	0	0	0	0	20766
AAV13	56711	11849	4585	15138	0
SAAV	581	0	0	0	0
BAAV	49	55127	0	1328	0
AAAV	140	0	0	0	0
Total	176457	176843	154677	168563	229623

After first round of screening in mice, AAV9 emerged more frequently at all five VR positions, with a predominating frequency of 71% at the position VR-VIII, a major receptor binding site.



C

HT2-Library

	VR4	VR5	VR6	VR7	VR8
AAV1	0	254	264	1213	3
AAV2	1748	0	0	0	866
AAV3	3	335	191	14	0
AAV4	43	43	5457	0	0
AAV5	0	0	0	1	1
AAV6	564	265	141	44	0
AAV7	0	0	174	288	113
AAV8	1199	128	277	212	1365
AAV9	1339	2910	2814	2836	5920
AAV10	34	684	0	3	587
AAV11	0	0	711	5058	2
AAV12	0	0	0	16	2
AAV13	5597	749	161	499	521
SAAV	297	0	0	0	0
BAAV	0	5827	5	70	0
AAAV	5	0	0	0	0
Total	10829	11195	10195	10254	9380

After two rounds of screening AAV13, BAAV, AAV4, AAV11, and AAV9 appeared more frequently at the positions VR-IV, -V, -VI, -VII, and VIII respectively

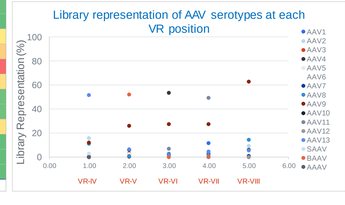


Figure 4. Occurrence and enrichment of AAV serotype specific VRs in the screening process. Tables showing the heat map illustrating the distribution of AAV serotypes across the five variable regions, accompanied by graphs illustrating the percentage representation of AAV serotypes at each variable region. A) Representation of AAV serotypes at each of the five variable regions in capsid variants - GHVR library, B) Representation of AAV serotypes at each of the five variable regions in capsid variants recovered from the first round of screening in mice, HT1 library, and C) Representation of AAV serotypes at each of the five variable regions in capsid variants recovered from the second round of screening in NHPs, HT2 library.

RESULTS, cont'd

Frequencies of Preferred AAV Serotypes Identified at the GH Loop Variable Regions During Screening Stages

Most frequently appeared AAV serotype at VR -IV to -VIII

			Mouse	NHP	
VR Region	Serotype	Library	1st Rd	2nd Rd	Top 40
VR-IV	AAV13	8.37	32.14	12.06	82.5
VR-V	BAAV	9.92	31.17	12.79	85
VR-VI	AAV4	10.51	31.63	11.85	80
VR-VII	AAV11	5.61	26.25	11.72	77.5
VR-VIII	AAV9	8.80	71.63	12.65	67.5

Table 1. Tables showing most frequently represented AAV serotypes at each of the five variable regions in the GH loop in the different stages of the screening and the top 40 capsid variants after the 2nd round of screening in the NHPs.

Frequency of AAV9, a Cardiotropic Serotype, at Five VR Positions During Screening Stages

Occurrence of AAV9 at VR -IV to -VIII

			Mouse	NHP	
VR Region	Serotype	Library	1st Rd	2nd Rd	Top 40
VR-IV	AAV9	6.37	34.34	2.87	10
VR-V	AAV9	7.07	48.52	6.65	2.5
VR-VI	AAV9	6.86	46.69	5.03	2.5
VR-VII	AAV9	9.07	57.55	6.06	5
VR-VIII	AAV9	8.80	71.63	12.68	67.5

Table 2. Tables showing the % occurrence of AAV9 variable regions at the five positions in the GH loop at different stages of the screening and the top 40 capsid variants after the 2nd round of screening in the NHPs.

CONCLUSION

We have developed an innovative library of AAV capsids through shuffling the GH loop variable regions (GHVR) and isolated a pool of novel capsids with the potential to deliver genes to cardiomyocytes efficiently

- Rationally designed an innovative library of AAV capsids via shuffling the five variable regions from 16 AAV serotypes on AAV9 capsid backbone.
- The GHVR library utilizes the amino acid sequences naturally evolved at the five variable regions which should provide key advantages:
  - Minimize the nonfunctional capsids
  - Eliminate premature stop codons in the variants.
- Sequential screening of the library in mice and cynomolgus monkeys yielded a pool of novel capsids with the potential to efficiently deliver genes to cardiomyocytes at lower doses

Identifying novel capsids through screening GHVR libraries constructed from capsids possessing desirable properties, such as low neutralizing antibody profiles, holds enormous potential for advancing gene therapy applications.