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Rational Design and Engineering of a Modified Adeno-Associated Virus (AAV1)-Based Vector System for Enhanced Retrograde Gene Delivery

BACKGROUND: After injection into muscle and peripheral nerves, a variety of viral vectors undergo retrograde transport to lower motor neurons. However, because of its attractive safety profile and durable gene expression, adeno-associated virus (AAV) remains the only vector to have been applied to the human nervous system for the treatment of neurodegenerative disease. Nonetheless, only a very small fraction of intramuscularly injected AAV vector arrives at the spinal cord.

OBJECTIVE: To engineer a novel AAV vector by inserting a neuronal targeting peptide (Tet1), with binding properties similar to those of tetanus toxin, into the AAV1 capsid.

METHODS: Integral to this approach was the use of structure-based design to increase the effectiveness of functional capsid engineering. This approach allowed the optimization of scaffolding regions for effective display of the foreign epitope while minimizing disruption of the native capsid structure. We also validated an approach by which low-titer tropism-modified AAV vectors can be rescued by particle mosaicism with unmodified capsid proteins.

RESULTS: Importantly, our rationally engineered AAV1-based vectors exhibited markedly enhanced transduction of cultured motor neurons, diminished transduction of nontarget cells, and markedly superior retrograde delivery compared with unmodified AAV1 vector.

CONCLUSION: This approach promises a significant advancement in the rational engineering of AAV vectors for diseases of the nervous system and other organs.

KEY WORDS: AAV-mediated gene delivery, Motor neuron disease, Rational design, Retrograde axonal transport, Vector engineering, Vector targeting

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Adeno-associated virus (AAV) has emerged as a promising vector for gene delivery because of its broad-tissue tropism, safety, ability to transduce both quiescent and dividing cells, and ability to mediate long-term gene expression. Recent isolation of novel AAV serotypes and advances in vector engineering have further enhanced the utility of these vectors. Gene therapy holds a variety of advantages for the treatment of a variety of neurological diseases. In several of these diseases, an optimal therapeutic response is facilitated by vectors that are capable of efficient retrograde axonal transport. In Parkinson disease, preservation and repair of the nigrostriatal pathway may require the expression of trophic genes in both the striatum (postsynaptic) and substantia nigra (presynaptic).[1] This type of targeting is facilitated by the ability of viral vectors to undergo retrograde axonal transport from the postsynaptic site into the presynaptic neurons. Similarly, retrograde transport can facilitate the delivery of genes to target neurons that are widely disbursed as in the case of treating corticospinal neurons to enhance spinal cord regeneration. Finally, retrograde vector

ABBREVIATIONS: AAV, adeno-associated virus; **ALS,** amyotrophic lateral sclerosis; **DMEM,** Dulbecco-modified Eagle medium; **DRG,** dorsal root ganglia; **DRP,** DNase-resistant particle; **MOI,** multiplicity of infection; **PBS,** phosphate-buffered saline; **PCR,** polymerase chain reaction; **rAAV,** recombinant adeno-associated virus

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delivery provides a minimally invasive means to access neurons in remote or sensitive structures, as is the case for dorsal root ganglia (DRG) in the treatment of neuropathy and pain or spinal cord motor neurons in the treatment of motor neuron diseases. Our laboratory has focused on both idiopathic and genetic motor neuron diseases. However, AAV-mediated retrograde gene delivery to motor neurons remains limited by inefficient axon binding and poor retrograde axonal transport.[2,3]

Previously, we isolated Tet1, a 12-mer linear peptide that binds selectively to differentiated pheochromocytoma (PC12) cells, primary motor neurons, and DRG in vitro.[4] We showed that Tet1 binds to the tetanus toxin GT1b receptor and described the in vivo neuronal binding properties and spinal cord uptake of Tet1 after peripheral delivery,[5] suggesting that insertion of this epitope into the AAV capsid might allow the generation of modified vectors with enhanced axon terminal binding and uptake. Although several studies have demonstrated genetic incorporation of peptide epitopes into the AAV2 capsid and subsequent modification of vector tropism,[6-9] our group has defined sites amenable to peptide insertion in other serotype AAV capsids[10] and has demonstrated that these peptide-modified vectors can also transduce target cells via these engineered interactions[10-13] Because AAV1 vectors have proven superior to AAV2 vectors for neuronal gene delivery and retrograde axonal transport, we sought to modify the AAV1 capsid by Tet1 peptide insertion.[2] Computational modeling was used to analyze capsid structure and conformation and to visualize and determine the optimal structural context for peptide display. This approach was instrumental in our ability to produce Tet1-modified AAV1 vectors and for these vectors to function properly and direct gene transduction to motor neurons.

Through this work, we have combined the goals and approaches of computational molecular design and capsid structure analysis to provide tools for the rational mutagenesis and functional modification of AAV vector particles. This approach used the analysis of 3-dimensional capsid structure to guide the selection of appropriate amino acid sequences to create a desired property or function. The convergence of high-speed computing, a tremendous increase in capsid structural information, and a growing understanding of the forces that control protein structure and maintain essential viral functions has resulted in dramatic advances in our ability to engineer protein function and structure and to create novel, rationally designed virus-based gene transfer vectors. Such an enhancement in our ability to engineer vectors for specific functions may prove critical to the practical application of gene transfer for a variety of therapeutic paradigms in addition to the studies described here for sensory and motor neuron gene delivery.

METHODS

Construction of Modified AAV Helper Plasmids

Modified AAV1 helper constructs encoding capsid proteins with Tet1 motif insertions were generated by polymerase chain reaction (PCR)-based site-directed mutagenesis as previously described.[10] Briefly, DNA primers were designed to encode the Tet1 motif and scaffolding sequences (Table) and used to direct PCR-based mutagenesis of the AAV helper plasmid pXR1.[14,15] This plasmid contains the entire AAV genome, encoding AAV2 Rep proteins and AAV1 Cap proteins, less the 2 viral inverted terminal repeats. PCR products were digested with *Dpn*I endonuclease to eliminate the parental plasmid template and were propagated in DH-5a bacteria (Invitrogen Life Technologies, Grand Island, New York). The nonhomologous linker sequences included in the PCR primers to encode the Tet1 scaffolding sequences were also designed to contain restriction sites. Therefore, mini-prep plasmid DNA could be extracted from ampicillin-resistant colonies and screened by restriction endonuclease digestion for confirmation of epitope insertion. Furthermore, all constructs were also subsequently sequenced to confirm epitope insertion and lack of second-site mutations.

Vectors

AAV1eGFP or AAV1RFP (dsRed2) vectors were produced by triple transfection as previously described.[10] To produce AAV vectors comprising Tet1-modified capsid proteins, HEK 293 cells were transfected with modified AAV helper plasmids, constructed as described above, in place of unmodified pXR1. In instances when mosaic particles were generated, unmodified pXR1 was included at a 20% molar equivalent of Tet1-modified AAV helper plasmid as described previously.[13] Transfections were carried out at 37°C with the use of the calcium phosphate transfection system (Invitrogen Life Technologies) according to the manufacturer's specifications. Forty-eight hours after transfection, cells were harvested by centrifugation at 500*g* for 10 minutes and resuspended in phosphate-buffered saline (PBS), and vector was released in 3 freeze-thaw cycles. The crude lysate was clarified by centrifugation at 500*g* 10 minutes, and viscosity was reduced by the addition of Benzonase (250 U/mL) and incubation at 37°C for 30 minutes. Lysate was then fractionated on an iodixanol step gradient[16] and further purified by high-performance liquid chromatography as described previously.[17] Final vector preparations were stored at −20°C in PBS containing 20% glycerol. DNase-resistant particle (DRP) values were determined by real-time PCR assay.[10]

Cell Lines

Low-passage-number (passage number 20-40) HEK 293 cells[18] and HeLa C12 cells[19] were grown in Dulbecco-modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL) at 37°C and 5% CO_2. PC12 pheochromocytoma cells were grown in DMEM supplemented with 10% horse serum, 5% fetal bovine serum, and penicillin (100 U/mL). For differentiation, cells were exposed for 2 to 3 days to 100 ng/mL of nerve growth factor (nerve growth factor 2.5S, Invitrogen

TABLE. Summary of Tet1 Insertion Mutants[a]

Vector Designation	Upstream Linker	Tet1 Peptide Epitope	Downstream Linker
AAV1.D590_P591insTet1a	AS	HLNILSTLWKYR	GLS
AAV1.D590insTet1b	ASDA	HLNILSTLWKYR	GLS
AAV1.D590_P591insTet1c	ASDA	HLNILSTLWKYR	ADGLS

[a] AAV, adeno-associated virus; AS, alanine-serine; GLS, glycine-leucine-serine.

Life Technologies) in DMEM with 2% horse serum and 1% fetal bovine serum. C2C12 cells[20] were cultured in Corning 6-well plates (Fisher, 07-200-80) with 1× DMEM (Gibco, 11965-092) supplemented with 10% Hyclone Cosmic Calf Serum (GE, SH30087.04IR) and 10 μg/mL ciprofloxacin.

Primary Cell Cultures

Spinal cords were obtained under sterile conditions from 15-day-old Sprague-Dawley rat embryos following an established protocol.[21] DRG and perineural membranes were removed, and cords were cut into 2-mm sections, which were then trypsinized. Cells were collected, centrifuged, pelleted, and then resuspended in complete growth medium made in supplemented Neurobasal Medium (Invitrogen Life Technologies). Cells were plated on glass coverslips in multiwell culture plates precoated with poly-L-lysine (Sigma-Aldrich, St. Louis, Missouri).

Campenot Chambers

Campenot Teflon chamber dividers (Tyler Research, Edmonton, Alberta, Canada) were carefully attached to Collagen/Matrigel-coated 35-mm culture dishes using silicone vacuum grease (Dow Corning, Midland, Michigan). Scratches were made in the Collagen/Matrigel with a pin rake. One drop of medium containing methylcellulose (1%) was placed onto the plate before the divider was set on the culture dish, which facilitated axon growth underneath the silicon grease barriers.[22] DRG explants were plated into one of the compartments of the chambers in a small volume of media and allowed to adhere for 2 hours. The compartment was then filled with growth medium. The adjacent compartments were filled with media supplemented with 100 ng/mL nerve growth factor to encourage neurite growth into these compartments.

Modeling

Modeling of modified AAV capsid structures was carried out first with SWISS-Model (http://swissmodel.expasy.org/)[23,24] and later with I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/).[25-27] The capsid protein was initially modeled as a monomer and extended to a trimer after automated modeling. SWISS- Model experienced difficulty in finding adequate loop structures for the Tet1c mutant in the automated mode and generally produced results that did not appear physiologically relevant even when using the alignment mode with a forced alignment to the relevant region of PDB 1LP3. I-TASSER successfully predicted several potential structures for each Tet1 structure with no constraints on the modeling. The SWISS-Model results and the I-TASSER results agreed on the probable structure of the inserted Tet1 sequence itself, including the location of the turn between the antiparallel β sheets. I-TASSER, however, positioned the Tet1 mutant insertions in physiologically realistic locations folded against the surface of the monomer, whereas SWISS-Model preferred to leave the insert as a free strand projecting into space. Although I-TASSER produced several potential results for each mutant, the majority of the structural differences between I-TASSER models were in the predicted structure of the N-terminal region of the protein, quite distant from the insert site. The position of the Tet1 mutants was generally as described in Figure 1, for which we used the highest-confidence I-TASSER-predicted result. The trimeric forms for the structures were constructed by structurally aligning the mutants with PDB 1LP3 and using the capsid-creation rotation symmetry matrixes from that file. The trimer was constructed of capsid subunits 0, 19, and 45. Only the Tet1a mutant required manual intervention, inversion of the β-turn curl direction, to avoid physiologically impossible topological conflicts. To increase our confidence that the folded positioning of the insert sequence was correct, we performed brief (40 000 time step, 2 femtoseconds per step) molecular minimizations of the trimer interface regions using NAMD (Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign).[28] Although insufficient to predict a lowest-energy conformation, our experience has been that these parameters are sufficient to detect dramatic spatial clashes caused by compositing independently modeled subunits. Each predicted mutant structure experienced some side-chain optimization during the minimization run, but none displayed symptoms of impossibly close van der Walls interactions or other signs of physical implausibility. We therefore believe that the modeled locations of the Tet1 mutants are physiologically reasonable possibilities for their placement.

Immunocytochemistry

Staining was performed with the neuronal cell marker Map-2. The protocol consisted of fixing the cell cultures for 40 minutes with 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) and blocking them with 0.1% Triton X-100 to 3% bovine serum albumin in PBS for 1 hour, before an overnight incubation with the primary antibodies. The next day, cells were washed 3 times with 1% bovine serum albumin/PBS and incubated for 1 hour with fluorochrome-conjugated secondary antibodies at room temperature. After washing, the coverslips were mounted on slides using Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, California).

Comparative Gene Transduction Assays

Titer-matched vector solutions (unmodified and Tet1-modified recombinant AAV1 [rAAV1]) were added to cultures and rocked gently to ensure equal distribution of the suspension over the cells. Typically, 3 wells per condition were used. Cells were fixed and processed for analyses 3 days after treatment. Images of transduced cells were acquired with a Nikon E400 microscope using a DS-Qi1 high-sensitivity cooled charge-coupled device camera and analyzed by use of the NIS-Elements imaging software (Nikon Instruments, Inc, Melville, New York) from 10 randomly selected fields per slide. The percentage of green fluorescent protein (GFP)- or red fluorescent protein-positive cells was determined by dividing the number of cell marker-positive, DAPI-positive cells that were also GFP- or red fluorescent protein-positive by the total number of cell marker-positive, DAPI-positive cells. Graphs of the relationship between dose and percentage of transduced cells were then generated. Data were expressed as mean ± SEM.

For quantification of gene transduction in Campenot chambers, the NIS-Elements imaging software was used to compare the fluorescence pixel intensity within the DRG cell bodies. By drawing a region of interest around the explants (white lines), we limited the area that was analyzed. Two channels of fluorescence were captured. The first channel was an internal control to compensate for variability of thickness, size, or density of cells within the explants. It consisted of DAPI staining (see **Figure A**, **Supplemental Digital Content 1**, http://links.lww.com/NEU/A693). The second channel recorded levels of fluorescence resulting from gene expression (see **Figure B**, **Supplemental Digital Content 1**, http://links.lww.com/NEU/A693), which was then expressed as a ratio of the internal control. The software calculated the area of pixel intensity within each region of interest (Table), and a graph was generated.

C2C12 cells at confluence were transduced with either control scrAAV1.eCBA.eGFP vector or scrAAV1tet1.eCBA.eGFP vector at

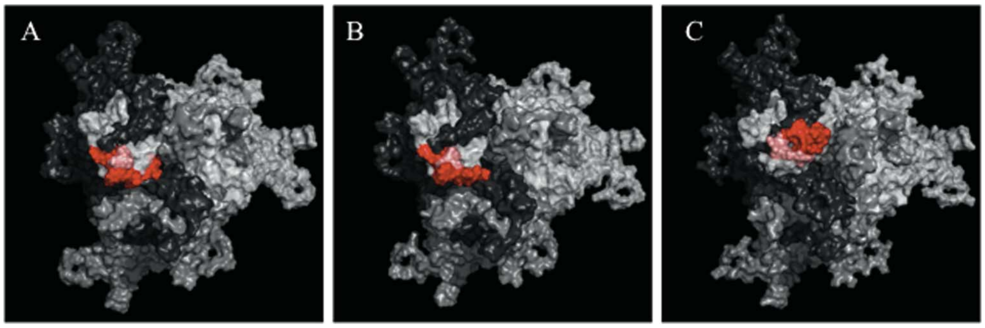

FIGURE 1. *A molecular modeling comparison of linker sequence effect on Tet1 peptide display and perturbation of native structure. The adeno-associated virus (AAV) capsid 3-fold axis of symmetry is depicted as a solvent exclusion surface, with each monomer a unique shade of gray, whereas the Tet1 peptide and linker sequences have added van der Walls spheres and are shaded red and pink, respectively.* **A**, *I-TASSER modeling of Tet1 with C-terminal linker alanine-serine (AS) and n-terminal linker glycine-leucine-serine (GLS; Tet1a) suggests that the peptide folds into the interface between and partially beneath the interlocking arms of each monomer that creates the 3-fold axis of symmetry of capsid.* **B**, *the C-terminal linker ASDA and N-terminal linker GLS (Tet1b) slightly change the side-chain polarity and presentation geometry of the peptide so that it is more accessible to the capsid exterior; however, it is still partially buried under the capsid surface.* **C**, *our modeling predicts that the C-terminal linker ASDA and N-terminal linker ADGLS (Tet1c) provide the best opportunity for maximum surface exposure of the Tet1 peptide on the exterior of the capsid with the least disruption of the native capsid structure.*

multiplicity of infection (MOI) of 10 000 and 33 000 in serum-free Gibco 1× DMEM. The cells were centrifuged at 1000 rpm for 30 minutes at room temperature and incubated for 4 hours at 37°C and 5% CO_2. At 4 hours after transduction, the wells were spiked to a final concentration of 2% serum and incubated at 37°C and 5% CO_2 for approximately 48 hours. The cells were rinsed with Gibco 1× Hanks balanced salt solution and harvested with Hyclone 0.05% trypsin containing 25 μmol/L EDTA. The trypsin was neutralized with Gibco 1× DMEM containing 10% Hyclone Cosmic Calf Serum and 10 μg/mL ciprofloxacin. The cells were centrifuged at 1000 rpm for 20 minutes and resuspended in Teknova TMN_{200}. Cells positive for GFP and mean florescence were determined by flow cytometry. Data were collected on a Becton Dickenson LSRII cytometer using BD FACSDiva software. A total of 30 000 events were collected for each sample, and viable cells were gated via a forward-scatter vs side-scatter plot. GFP-positive cells were subsequently gated from the viable cell population. Voltages were set based on autofluorescence of unlabeled cells.

Statistical Analysis

Cell counts were performed in 10 different fields per slide. All experiments were performed in triplicate. The effects of vector treatment and MOI were compared by use of 2-way analysis of variance; however, Tukey tests also were performed, and the data are presented as 2-way analysis of variance with the *P* values from the Tukey tests ($P < .05$).

RESULTS

Generation of AAV Capsids Containing Tet1 Peptide Insertions

We have previously shown that AAV1 can tolerate the insertion of exogenous peptides after VP1 amino acid 590.[29] Importantly, we have also shown that the inserted sequences can be displayed on the surface of assembled AAV particles and can promote novel capsid-protein interactions.[13,29] To use these findings for the purpose of generating Tet1-modified AAV particles, we introduced oligonucleotides encoding the Tet1 motif into the Cap ORF of the AAV1 helper plasmid pXR1[14] by PCR-based site-directed mutagenesis, creating pXR1-Cap1.D590_P591insTet1a. Short peptide linkers, previously optimized for the display of heterologous ligands,[11,13] were also included in an attempt to maintain local capsid flexibility and to promote efficient display of the Tet1 epitope on the surface of the assembled AAV vector particles (Table). In the first instance, these were made up of an alanine-serine upstream linker and a glycine-leucine-serine downstream linker flanking the Tet1 epitope (Tet1a designation, Table).

Tet1a-Modified AAV1 Capsid Proteins Inefficiently Package Vector Genomes and Fail to Mediate Efficient Gene Transfer

To assess the impact of the Tet1a modification on DNA packaging and infectivity, we determined particle and infectious titers of AAV1 and Tet1a-modified AAV1, AAV1.D590_P591ins-Tet1a, vectors (Table). A real-time PCR assay was used to assess DRP titer,[30] and transduction of HeLa C12 cells was performed to assess particle infectivity. Compared with unmodified AAV1 capsids, the production of AAV1.D590_P591insTet1a capsids was 66.5-fold less efficient ($5.95 \pm 0.41 \times 10^{10}$ DRP per 1 mL for AAV1.D590_P591insTet1a vs $3.96 \pm 2.2 \times 10^{12}$ DRP per 1 mL for AAV1). Similarly, transduction of HeLa C12 cells was significantly impaired by roughly 3 orders of magnitude by the Tet1 modification (see **Figure**, **Supplemental Digital Content 2**, http://links.lww.com/NEU/A694).

Computational Modeling of Tet1-Modified AAV1 Capsid Proteins

We hypothesized that conformational stresses imposed on the AAV1 capsid by the Tet1 peptide insertion might be responsible for the observed decreases in titers. We therefore pursued 2 complementary approaches to rescue titer of peptide-modified AAV1 particles. First, we modeled the structure of the AAV1 capsid at the 3-fold axis of symmetry to see if sequences flanking the Tet1 insert could be altered either to minimize distortion of the native capsid structure or to promote more efficient display of the targeting epitope. Modeling preformed with both I-TASSER and SWISS-Model and validated with molecular dynamics simulations in NAMD indicated that the Tet1 peptide favorably forms a pair of antiparallel β strands. Postulating that changing the length of the linker sequences and the linker side chain polarity might predispose this short β sheet motif toward different positioning on the capsid surface, we modeled the structures of 2 additional Tet1 peptide modifications with different linker/scaffolding sequences (Tet1b and Tet1c; Table). When flanked by alanine and serine at the C-terminus and glycine, leucine, and serine at the N-terminus, as in the original Tet1a construct, the Tet1 epitope folded "downward" and packed between and under the VP3 monomer arms that create the 3-fold axis of symmetry. This not only might effectively shield the epitope from any interaction with its targeted receptor but also appeared to distort the native capsid structure at the 3-fold axis of symmetry (Figure 1A). Modification of the C-terminal linker to include aspartic acid and alanine allowed slightly better presentation of Tet1, allowing it to stack alongside the monomer arm into which it was inserted; however, it was still partially buried (Figure 1B). However, the Tet1c modification, which includes additional alanine and aspartic acid residues in the N-terminal linker sequence, allowed the epitope to lie on top of the arm into which it was inserted, resulting in unobstructed display on the capsid surface (Figure 1C). In fact, modeling of the Tet1c modification predicted the presentation of a "looped" version of the Tet1 epitope, with the C-terminal and N-terminal residues of the inserted sequences in close proximity to each other. Insertions such as this, which minimize the induced gap in and therefore might minimize distortion of the native structure, although facilitating more effective presentation of the targeting epitope, could potentially increase both particle titer and the ability of the modified particles to interact with their targeted receptors.

Efficient Production of Mosaic Tet1c-Modified Vectors Capable of Mediating Specific Gene Transfer to Differentiated Pheochromocytoma (PC12) Cells

Based on our computational modeling, we generated 2 additional packaging constructs, pXR1- Cap1.D590_P591insTet1b and pXR1-Cap1.D590_P591insTet1c, for building the modified Tet1b and Tet1c sequences into the AAV1 capsid. As suggested above and described previously by our group,[13] these constructs were used with AAV helper plasmid pXR1, encoding unmodified AAV1 capsid proteins, to generate mosaic AAV particles containing different ratios of Tet1-modified and unmodified capsid proteins. Particle titers were determined by real-time PCR, and gene transduction was assessed on HEK 293 and differentiated pheochromocytoma (PC12) cells. Mosaic particles generated from a mixture of 80% Tet1c-modified pXR1 plasmid (pXR1-Cap1.D590_P591insTet1c) and 20% unmodified pXR1 plasmid into HEK 293 packaging cells were produced 4-fold more efficiently than particles made up entirely of Tet1c-modified capsid proteins ($P < .05$), and significant increases in particle titer were realized with the use of optimized linker/scaffolding sequences flanking Tet1 ($P < .05$; Figure 2). These findings validate the ability of particle mosaicism to rescue titer of some tropism-modified AAV vector particles, as well as our computational modeling approach to rationally engineer modified AAV capsids. However, the real benefit of this strategy was evidenced by the ability of Tet1c-modified mosaic AAV1 vectors (Tet1c-rAAV) to mediate significantly enhanced transduction of PC12 cells and significantly lower transduction of HEK 293 cells (Figure 3). Transduction of HEK 293 cells at 100 DRPs per cell decreased >55-fold from 32.32 ± 6.98% (mean ± SEM) to only 0.58 ± 1.29% ($P < .05$), whereas at 1000 DRPs per cell, transduction decreased from

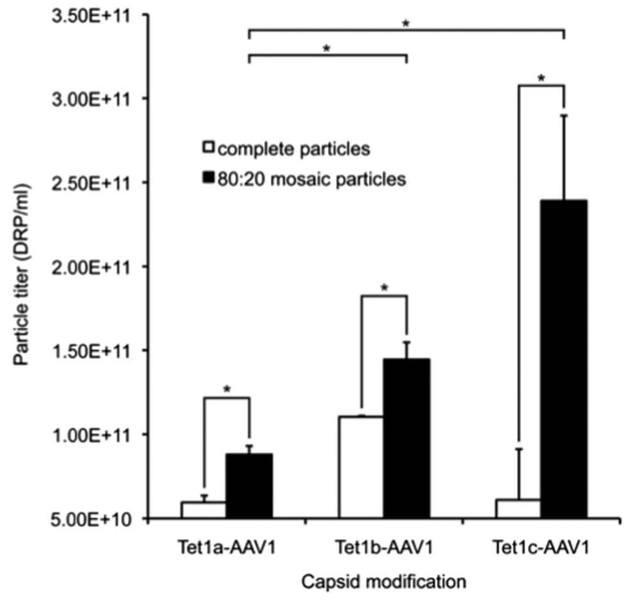

FIGURE 2. *Particle titers of Tet1-modified recombinant adeno-associated virus 1 (rAAV1) vectors. DNase-resistant particle (DRP) titers were determined by real-time polymerase chain reaction assay.[30] Particles comprising entirely Tet1-modified AAV1 capsids proteins are compared with mosaic particles assembled from HEK 293 cells transfected with an 80:20 mixture of plasmid DNA encoding Tet1-modified AAV1 capsid proteins and unmodified AAV1 capsid proteins. All data are shown as the mean of triplicate determinations. Bars indicate the standard error of the mean. A 2-way analysis of variance test revealed that a significant effect depended on both capsid mosaicism and linker/scaffolding sequences flanking the Tet1 epitope (*P < .05).*

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