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Mechanistic Insights into the Enhancement of Adeno-Associated Virus Transduction by Proteasome Inhibitors

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Proteasome inhibitors (e.g., bortezomib, MG132) are known to enhance adeno-associated virus (AAV) transduction; however, whether this results from pleotropic proteasome inhibition or off-target serine and/or cysteine protease inhibition remains unresolved. Here, we examined recombinant AAV (rAAV) effects of a new proteasome inhibitor, carfilzomib, which specifically inhibits chymotrypsin-like proteasome activity and no other proteases. We determined that proteasome inhibitors act on rAAV through proteasome inhibition and not serine or cysteine protease inhibition, likely through positive changes late in transduction.

Adeno-associated virus (AAV) is frequently utilized as a gene delivery vector for clinical application; thus, several approaches have been undertaken to increase efficacy, including transgene optimization (1–3), capsid alteration (reviewed in reference 4), and drug treatments to enhance transduction (5–13). Proteasome inhibitors (PIs) were first described to enhance recombinant AAV (rAAV) polarized airway cell transduction (6), and since then PIs, including *N*-acetyl-L-leucinyl-L-leucinyl-norleucinal (LLnL) (6, 14–19), MG132 (5, 6, 8, 11, 14, 17, 20–24), bortezomib (11, 25), and celastrol (26), have been observed to enhance transduction in many cell types both *in vitro* and *in vivo*. Nevertheless, questions remain regarding the mechanism of this enhancement. Although ubiquitinated rAAV2 capsid proteins accumulate after PI treatment, suggesting PIs prevent the degradation of ubiquitinated AAV capsids and lead to increased transgene expression, some level of capsid dissociation (6) or phosphorylation (27, 28) appears to be necessary for ubiquitination, and the role of the proteasome in these effects has not been directly examined. In addition to proteasome inhibition, PIs are commonly observed to inhibit other proteases, such as cysteine (MG-132) and serine (MG-132 and bortezomib) proteases (29). These proteases have very different cellular roles from the proteasome, which degrades ubiquitinated cytoplasmic and nuclear proteins, including lysosomal degradation and calcium-dependent intracellular signaling. In fact, the *in vivo* peripheral neuropathy caused by bortezomib is the result of serine protease inhibition leading to neurotoxicity (30), demonstrating the importance of off-target effects with clinically relevant dosing of PIs. The broad range of inhibition caused by PIs has caused many in the field of rAAV research to hypothesize that the effects of PIs on rAAV transduction are due to off-target effects of PIs and not inhibition of the proteasome. In addition, whether the enhancement of rAAV transduction occurs through proteasome inhibition or protease inhibition, it is also unclear whether the effects of PIs prevent the degradation of rAAV virions or whether they cause a positive change in transduction.

The promiscuity of so-called "first-generation" PIs (i.e., those available before carfilzomib) led to the development of new PIs with restricted specificity. Proteases, including the proteasome, act through a nucleophilic attack by their active site residue, which can be serine, cysteine, or threonine, or by water in the case of aspartic and metalloproteases. The protease's active site residue is used to classify the protease (e.g., serine protease). Unlike other classes of proteases, active site threonine of the proteasome is the N-terminal residue of each catalytic subunit, exposing the amino group to possible reactivity (31). Carfilzomib, a second-generation PI, relies on this amino group to form a morpholino, covalently inhibiting cleavage (32), and so cannot inhibit other proteases (33, 34). In fact, carfilzomib highly inhibits only the chymotrypsin-like activity of the proteasome (34), making it a useful tool for examining the importance of proteasome inhibition on enhancement of rAAV transduction and addressing the hypothesis stated above that PIs act on rAAV transduction through off-target effects on other proteases. To determine whether the enhancement of rAAV transduction observed with PI treatment occurs from proteasome inhibition or from inhibition of other proteases, we utilized several PIs as well as cysteine and serine protease inhibitors and assessed their effect on rAAV transduction.

Carfilzomib enhances rAAV2 transduction *in vitro*. To address the question of whether a specific PI is sufficient to enhance rAAV transduction, we utilized three PIs, MG132, bortezomib, and carfilzomib (Selleck Chemicals), and an rAAV serotype 2 (rAAV2) vector expressing luciferase or enhanced green fluorescent protein (EGFP) transgenes (35). We coadministered the drugs with 1,000 vector genomes per cell (vg/cell) rAAV2 to HeLa cells and compared their effects on transduction at 24 h (8). Using luciferase vector, we determined that all of these PIs enhanced rAAV2 transduction at a range of doses, although we observed shifts in the curves that correlate with differing 50% inhibitory concentration (IC_{50}) values (bortezomib, 0.6 nM; carfilzomib, 5 nM; and MG132, 100 nM) (36–38) (Fig. 1A). To differentiate between the numbers of cells transduced and the level of their transduction, we treated with 1 μM PI, transduced with 500 vg/

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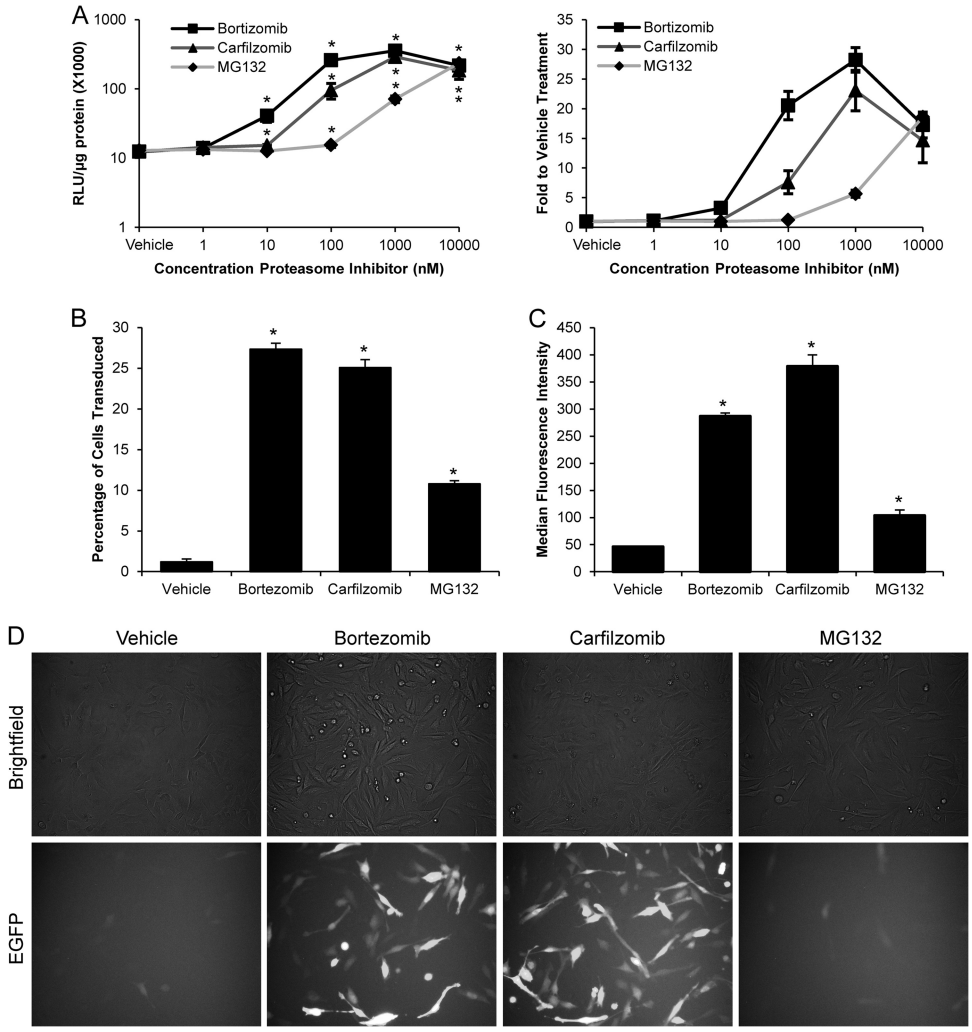

FIG 1 Carfilzomib enhances rAAV2 transduction. (A) HeLa cells were cotreated with the indicated dose of bortezomib, carfilzomib, MG132, or a dimethyl sulfoxide (DMSO) vehicle control and transduced with 1,000 vg/cell rAAV2-luciferase. Transduction at 24 h is indicated as normalized luciferase activity and fold values to the vehicle-treated group. (B and C) HeLa cells were cotreated with 1 μM PI and 500 vg/cell rAAV2-EGFP, and transduction was analyzed by flow cytometry at 24 h. The percentage of cells transduced (B) and median fluorescence intensity of the transduced cells (C) are indicated. (D) Bright-field and EGPF fluorescence images at 24 h postransduction of cells treated as described for panels B and C, visually indicating transduction. Data shown are representative of three independent experiments. Error bars represent one standard deviation (SD). *, $P < 0.05$ versus the vehicle control based on the Kruskal-Wallis test.

cell rAAV2-EGFP, and assayed EGFP expression by flow cytometry (10). The PIs enhanced both the percentage of cells transduced (Fig. 1B) and their fluorescence intensity (Fig. 1C). This enhancement can also be observed visually (Fig. 1D). Carfilzomib's transduction enhancement suggests that proteasome inhibition is sufficient for PI effects on rAAV transduction, as this is carfilzomib's only activity. Furthermore, the similar enhancement observed between bortezomib and carfilzomib suggests that the enhancement from bortezomib may be due primarily to proteasome inhibition.

Serine and cysteine protease inhibition does not enhance rAAV2 transduction. As we found proteasome inhibition sufficient for the enhancement of rAAV transduction, we asked whether serine protease inhibition, observed with MG132 and bortezomib, or cysteine protease inhibition, observed with MG132, have effects on rAAV2 transduction. We treated HeLa cells twice with phenylmethanesulfonyl fluoride (PMSF) to inhibit serine proteases as has been described (39), coadministered 1,000 vg/cell rAAV2 with the second dose, and analyzed transduction by luciferase assay at 24 h. We observed no increases in rAAV2 transduction from treatment with a 1,000-fold range of PMSF doses with a maximum dose 10-fold over PMSF's working concentration (Fig. 2A), suggesting that serine protease inhibition does not enhance rAAV2 transduction. We confirmed the ability of PMSF to inhibit serine proteases at these concentrations with a colorimetric trypsin activity assay (BioVision Inc.), which measured cleavage of a trypsin substrate over time (Fig. 2B). To investigate whether cysteine proteases affect rAAV transduction, we treated cells with E-64 and assayed transduction as described above. rAAV2 transduction did not change over a 10,000-fold range of E-64 doses with a maximum dose 10- to 100-fold over E-64's working concentration (Fig. 2C), suggesting that cysteine protease inhibition also does not enhance rAAV2 transduction. We confirmed the ability of E-64 to inhibit cysteine proteases at

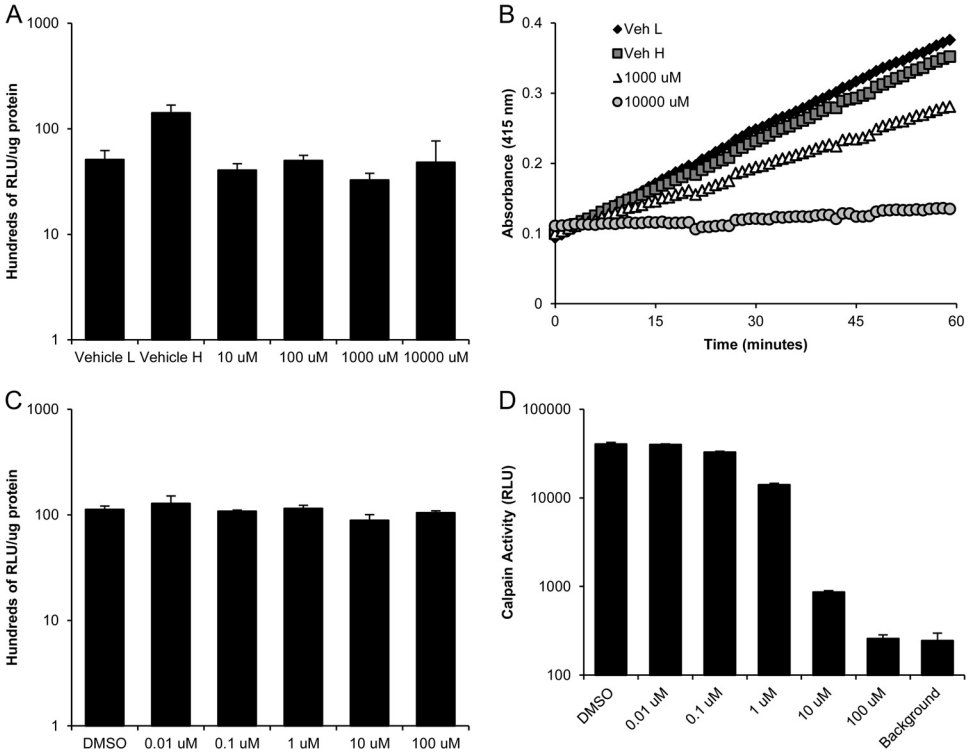

FIG 2 Serine and cysteine protease inhibition does not enhance rAAV2 transduction. (A) HeLa cells were treated 3 h prior to and at the time of transduction with the indicated dose of PMSF, a serine protease inhibitor, or an ethanol vehicle control and transduced with 1,000 vg/cell rAAV2-luciferase. Transduction is indicated as normalized luciferase activity. The "Vehicle L" group corresponds to treatments with 10 to 1,000 μM, while the "Vehicle H" group corresponds to treatment with 10,000 μM. (B) Indicated concentrations of PMSF or vehicle were combined with 0.002% trypsin and incubated at 30 min at room temperature. Solutions were diluted 1:10 in assay buffer and combined with trypsin substrate in quadruplicate. Average absorbance at 415 nm is shown for 60 readings at 60-s intervals. (C) HeLa cells were treated as described for panel A, with E-64, a cysteine protease inhibitor, or a DMSO vehicle control. Transduction is indicated as normalized luciferase activity. (D) Indicated concentrations of E-64 or vehicle control were combined with 20 nM human calpain 1 (BioVision), incubated at room temperature for 10 min, and combined with Calpain-Glo luciferase reagent (Promega) with 2 mM $CaCl_2$. E-64 activity is indicated as relative light units. Data shown are representative of three independent experiments. Error bars represent one SD.

these concentrations with a luminescent calpain assay (Promega), which measured cleavage of a luminescent substrate in the presence and absence of E-64 (Fig. 2D). Although cathepsins B and L (cysteine proteases) have been suggested to be important for rAAV transduction (40), we also observed no decreases in transduction with E-64 treatment. This may be due to a difference in species, as the interaction of cathepsins with rAAV was identified in murine cells, whereas we are using human cells. Nevertheless, as PI inhibition of these proteases would only decrease transduction, cysteine protease inhibition is unlikely to be the mechanism by which PIs enhance rAAV transduction. Taken together, these data suggest that enhancement of rAAV transduction by PIs is not due to off-target effects on other proteases.

Bortezomib and carfilzomib act on rAAV transduction through the same mechanism. Our results thus far suggest that proteasomal inhibition is responsible for enhancement of rAAV transduction after PI treatment. To investigate this hypothesis further, we determined whether bortezomib and carfilzomib are both effective on several different AAV serotypes. We treated HeLa cells with 1 μM bortezomib or carfilzomib and 20,000 vg/cell rAAV6, 100,000 vg/cell rAAV8, or 100,000 vg/cell rAAV9 and assayed transduction by flow cytometry at 24 h. The enhancement of the percentage of cells transduced was similar in all serotypes between bortezomib and carfilzomib, although carfilzomib enhanced fluorescence intensity more for some serotypes (Fig. 3A), strengthening the hypothesis that bortezomib and carfilzomib act through the same mechanism. Furthermore, to our knowledge, this is the first report of rAAV9 enhancement by PIs. To assess directly whether bortezomib and carfilzomib act through the same mechanism, we performed an exchange experiment where we treated cells with bortezomib or carfilzomib in combination, transduced with rAAV2 as before, and assayed transduction at 24 h. There were no increases or decreases in transduction from combining these two drugs (Fig. 3B), suggesting they can be used interchangeably. Combined with our other data, this suggests that bortezomib and carfilzomib both act to enhance rAAV transduction through proteasome inhibition.

Consequently, two hypotheses can be drawn for how proteasome inhibition enhances rAAV transduction: (i) proteasome inhibition prevents the degradation of rAAV capsids, increasing the rAAV pool available to complete transduction; (ii) as misfolded protein responses can enhance rAAV transduction (41), a general misfolded protein response and/or ubiquitination of rAAV capsids facilitates late steps in transduction. To address these hypotheses, we treated HeLa cells with bortezomib or carfilzomib and rAAV2 as described before and assayed intracellular vector genome copy number at 24 h by quantitative PCR (qPCR) (10). The copy number was increased 2.3-fold and 1.8-fold by bortezomib

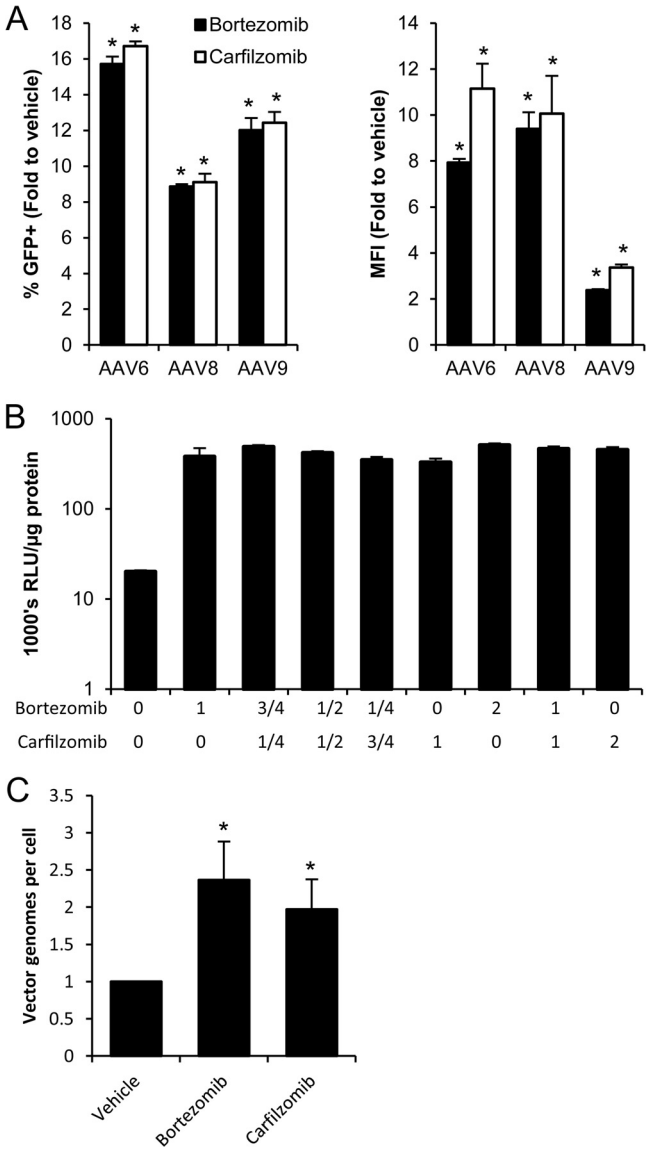

FIG 3 Bortezomib and carfilzomib act on rAAV2 transduction through the same mechanism. (A) HeLa cells were treated with 1 μM bortezomib, carfilzomib, or vehicle control and 20,000 vg/cell rAAV6-EGFP, 100,000 vg/cell rAAV8-EGFP, or 100,000 vg/cell rAAV9-EGFP, and transduction was assayed by flow cytometry at 24 h postransduction. Transduction is indicated as fold values of percentages of cells transduced and median fluorescence intensities of vehicle control groups. (B) HeLa cells were treated with the indicated doses (μM) of bortezomib and carfilzomib or a vehicle control and 1,000 vg/cell rAAV2-luciferase. Transduction is indicated as normalized luciferase activity. (C) HeLa cells were treated as described for Fig. 1B, and intracellular vector genome copy number was analyzed at 24 h postransduction by qPCR. Data are indicated as fold values of vg/cell of the vehicle control. Data shown in panels A and B are representative of three independent experiments. Data shown in panel C are the means from three independent experiments. Error bars represent one SD. *, $P < 0.05$ versus the vehicle control group based on the Kruskal-Wallis test.

and carfilzomib treatment, respectively (Fig. 3C); however, this was much smaller than the 28-fold and 23-fold transduction increases observed with bortezomib and carfilzomib (Fig. 1A). These data suggest the enhancement of transduction observed is unlikely to be directly due to capsid retention (hypothesis i). Instead, it is more likely that the buildup of ubiquitinated capsid or a misfolded protein response led to increased favorability in late transduction steps. This agrees with previous results demonstrating increased nuclear localization of virus following PI treatment (6, 11, 17, 23, 26, 27) and specifically increased nucleolar localization (8). Furthermore, we previously reported that treatment with arsenic trioxide leads to increased transduction through stabilization of perinuclear rAAV capsids and that this effect was distinguishable from PI effects (10). Taken with our current results, this suggests that, while arsenic trioxide is directly influencing transduction through the retention of capsids that would otherwise be degraded, PIs are influencing transduction through a modification secondary to degradation, explaining their differing effects.

Carfilzomib is less successful at enhancing rAAV transduction *in vivo* than bortezomib. As carfilzomib and bortezomib demonstrate similar rAAV transduction enhancement *in vitro*, we tested carfilzomib's ability to enhance rAAV2 transduction *in vivo* to determine whether proteasome inhibition is sufficient for enhancement of rAAV transduction *in vivo*. We utilized rAAV2, a liver-tropic vector, as the majority of AAV biology has been studied with this serotype; however, pharmacokinetic studies with carfilzomib demonstrate low activity in the liver due to drug metabolism (34). Therefore, we expected less enhancement of rAAV transduction in the liver with carfilzomib than with bortezomib. All *in vivo* experiments were approved by and conducted in accordance with the policies of the University of North Carolina at Chapel Hill's Institutional Animal Care and Use Committee. We coadministered either 0.5 mg bortezomib/kg of body weight or 1 mg carfilzomib/kg, a similar molar dose, and 1×10^{11} vg/mouse rAAV2 retro-orbitally into age-matched female BALB/c mice (Jackson Laboratories) (11) and assayed transduction through live luciferase imaging (10). No acute liver toxicity occurred with the vehicle or either of the proteasome inhibitors at this dose (Fig. 4A). We observed enhanced transduction from both bortezomib and carfilzomib treatment at 7 days postransduction (Fig. 4B), which quantified as 12.4-fold and 2.7-fold enhancements, respectively (Fig. 4C). At day 14, bortezomib mice maintained higher transduction than vehicle mice; however, carfilzomib and vehicle mice demonstrated similar transduction (Fig. 4D). *Ex vivo* quantification of transduction by luciferase assay and vector genome copy number (10) confirmed the live imaging data (Fig. 4E). Despite the expected lesser effects of carfilzomib than of bortezomib in the liver, these data demonstrate that proteasome inhibition is sufficient for the enhancement of rAAV transduction *in vivo*. In addition, this suggests that, although carfilzomib is not ideal for *in vivo* rAAV transduction enhancement in the liver, other second-generation, highly specific PIs should be evaluated for this purpose as they become available.

Conclusions. Overall, our data demonstrate that proteasome inhibition is sufficient for rAAV transduction enhancement, and serine and cysteine protease inhibition is unlikely to contribute to this enhancement. These data will alter the prevailing view in the field that the PIs act on rAAV transduction through off-target effects and instead demonstrate that they act through inhibition of the proteasome. Furthermore, the strategies employed to address these questions could now be applied to other viruses which are thought to be affected by proteasomal activity, such as hepatitis B virus or herpes simplex virus (42, 43). Additionally, the transduction increase seems to be secondary to prevention of rAAV capsid degradation and is instead due to a positive change in late stages of transduction. Furthermore, although carfilzomib is not ideal for

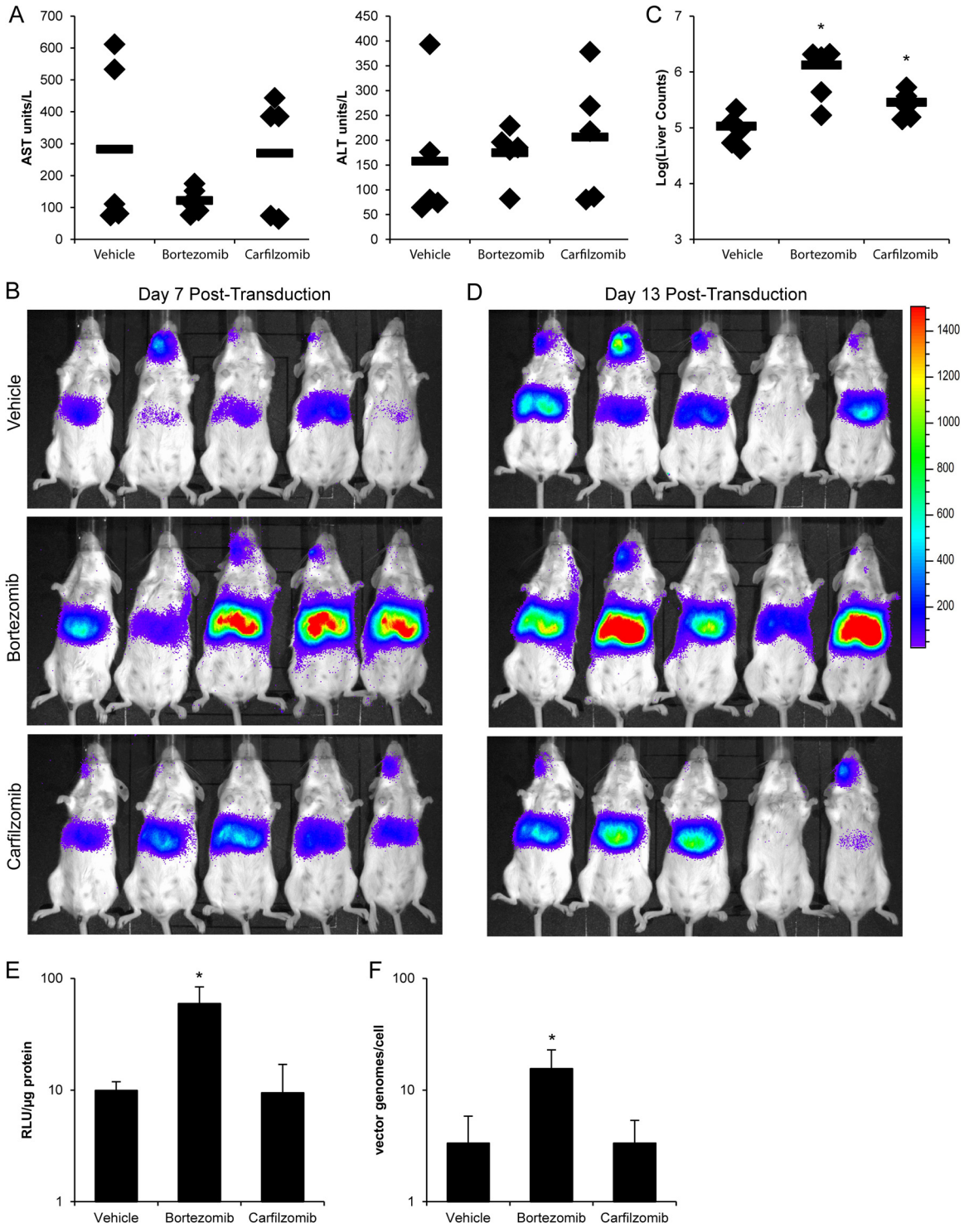

FIG 4 Bortezomib is more efficient at enhancing rAAV2 transduction *in vivo* than carfilzomib. Female BALB/c mice were treated with 1E11 vg/mouse rAAV2-luciferase and 0.5 mg bortezomib/kg, 1 mg carfilzomib/kg, or DMSO vehicle control. (A) Serum was collected from mice at 24 h posttreatment, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured. Individual animals are indicated by diamonds, and the mean is indicated by bars. Transduction was assayed by live imaging at 7 days postransduction (B), and light output from the area of the liver was quantified (C). (D) Transduction at 13 days was assayed by live imaging. (E) At 14 days, livers were harvested; transduction is indicated by normalized luciferase activity. (F) Vector genome copy number was assayed by qPCR. Error bars represent one SD. *, $P < 0.05$ versus the vehicle control based on the Kruskal-Wallis test.

enhancing rAAV-mediated liver transduction, our data suggest that other second-generation PIs in development, such as ONX0912, MLN9708, and marizomib (44), should be examined for enhancement of rAAV transduction *in vivo*. As these PIs may have fewer side effects than bortezomib, this may become important for the enhancement of rAAV clinical gene therapy.

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REFERENCES

1. **Binny C, McIntosh J, Della Peruta M, Kymalainen H, Tuddenham EG, Buckley SM, Waddington SN, McVey JH, Spence Y, Morton CL, Thrasher AJ, Gray JT, Castellino FJ, Tarantal AF, Davidoff AM, Nathwani AC.** 2012. AAV-mediated gene transfer in the perinatal period results in expression of FVII at levels that protect against fatal spontaneous hemorrhage. Blood **119:**957–966.
2. **Goodrich LR, Phillips JN, McIlwraith CW, Foti SB, Grieger JC, Gray SJ, Samulski RJ.** 2013. Optimization of scAAVIL-1ra in vitro and in vivo to deliver high levels of therapeutic protein for treatment of osteoarthritis. Mol. Ther. Nucleic Acids **2:**e70.
3. **Markusic DM, Herzog RW.** 2012. Liver-directed adeno-associated viral gene therapy for hemophilia. J. Genet. Syndr. Gene Ther. **1:**1–9.
4. **Mitchell AM, Nicolson SC, Warischalk JK, Samulski RJ.** 2010. AAV's anatomy: roadmap for optimizing vectors for translational success. Curr. Gene Ther. **10:**319–340.
5. **Douar AM, Poulard K, Stockholm D, Danos O.** 2001. Intracellular trafficking of adeno-associated virus vectors: routing to the late endosomal compartment and proteasome degradation. J. Virol. **75:**1824–1833.
6. **Duan D, Yue Y, Yan Z, Yang J, Engelhardt JF.** 2000. Endosomal processing limits gene transfer to polarized airway epithelia by adeno-associated virus. J. Clin. Invest. **105:**1573–1587.
7. **Ferrari FK, Samulski T, Shenk T, Samulski RJ.** 1996. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. J. Virol. **70:**3227–3234.
8. **Johnson JS, Samulski RJ.** 2009. Enhancement of adeno-associated virus infection by mobilizing capsids into and out of the nucleolus. J. Virol. **83:**2632–2644.
9. **Ju XD, Lou SQ, Wang WG, Peng JQ, Tian H.** 2004. Effect of hydroxyurea and etoposide on transduction of human bone marrow mesenchymal stem and progenitor cell by adeno-associated virus vectors. Acta Pharmacol. Sin. **25:**196–202.
10. **Mitchell AM, Li C, Samulski RJ.** 2013. Arsenic trioxide stabilizes accumulations of adeno-associated virus virions at the perinuclear region, increasing transduction *in vitro* and *in vivo*. J. Virol. **87:**4571–4583.
11. **Monahan PE, Lothrop CD, Sun J, Hirsch ML, Kafri T, Kantor B, Sarkar R, Tillson DM, Elia JR, Samulski RJ.** 2010. Proteasome inhibitors enhance gene delivery by AAV virus vectors expressing large genomes in hemophilia mouse and dog models: a strategy for broad clinical application. Mol. Ther. **18:**1907–1916.
12. **Prasad KM, Xu Y, Yang Z, Toufektsian MC, Berr SS, French BA.** 2007. Topoisomerase inhibition accelerates gene expression after adeno-associated virus-mediated gene transfer to the mammalian heart. Mol. Ther. **15:**764–771.
13. **Russell DW, Alexander IE, Miller AD.** 1995. DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. Proc. Natl. Acad. Sci. U. S. A. **92:**5719–5723.
14. **Yan Z, Zak R, Luxton GW, Ritchie TC, Bantel-Schaal U, Engelhardt JF.** 2002. Ubiquitination of both adeno-associated virus type 2 and 5 capsid proteins affects the transduction efficiency of recombinant vectors. J. Virol. **76:**2043–2053.
15. **Ding W, Yan Z, Zak R, Saavedra M, Rodman DM, Engelhardt JF.** 2003. Second-strand genome conversion of adeno-associated virus type 2 (AAV-2) and AAV-5 is not rate limiting following apical infection of polarized human airway epithelia. J. Virol. **77:**7361–7366.
16. **Yan Z, Zak R, Zhang Y, Ding W, Godwin S, Munson K, Peluso R, Engelhardt JF.** 2004. Distinct classes of proteasome-modulating agents cooperatively augment recombinant adeno-associated virus type 2 and type 5-mediated transduction from the apical surfaces of human airway epithelia. J. Virol. **78:**2863–2874.
17. **Denby L, Nicklin SA, Baker AH.** 2005. Adeno-associated virus (AAV)-7 and -8 poorly transduce vascular endothelial cells and are sensitive to proteasomal degradation. Gene Ther. **12:**1534–1538.
18. **Grieger JC, Samulski RJ.** 2005. Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. J. Virol. **79:**9933–9944.
19. **Li W, Zhang L, Wu Z, Pickles RJ, Samulski RJ.** 2011. AAV-6 mediated efficient transduction of mouse lower airways. Virology **417:**327–333.
20. **Liu Y, Joo KI, Wang P.** 2012. Endocytic processing of adeno-associated virus type 8 vectors for transduction of target cells. Gene Ther. **20:**308–317.
21. **Pajusola K, Gruchala M, Joch H, Luscher TF, Yla-Herttuala S, Bueler H.** 2002. Cell-type-specific characteristics modulate the transduction efficiency of adeno-associated virus type 2 and restrain infection of endothelial cells. J. Virol. **76:**11530–11540.
22. **Hacker UT, Wingenfeld L, Kofler DM, Schuhmann NK, Lutz S, Herold T, King SB, Gerner FM, Perabo L, Rabinowitz J, McCarty DM, Samulski RJ, Hallek M, Buning H.** 2005. Adeno-associated virus serotypes 1 to 5 mediated tumor cell directed gene transfer and improvement of transduction efficiency. J. Gene Med. **7:**1429–1438.
23. **Jennings K, Miyamae T, Traister R, Marinov A, Katakura S, Sowders D, Trapnell B, Wilson JM, Gao G, Hirsch R.** 2005. Proteasome inhibition enhances AAV-mediated transgene expression in human synoviocytes in vitro and in vivo. Mol. Ther. **11:**600–607.
24. **Cheng B, Ling C, Dai Y, Lu Y, Glushakova LG, Gee SW, McGoogan KE, Aslanidi GV, Park M, Stacpoole PW, Siemann D, Liu C, Srivastava A.** 2012. Development of optimized AAV3 serotype vectors: mechanism of high-efficiency transduction of human liver cancer cells. Gene Ther. **19:**375–384.
25. **Nathwani AC, Cochrane M, McIntosh J, Ng CY, Zhou J, Gray JT, Davidoff AM.** 2009. Enhancing transduction of the liver by adeno-associated viral vectors. Gene Ther. **16:**60–69.
26. **Zhang FL, Jia SQ, Zheng SP, Ding W.** 2011. Celastrol enhances AAV1-mediated gene expression in mice adipose tissues. Gene Ther. **18:**128–134.
27. **Zhong L, Zhao W, Wu J, Li B, Zolotukhin S, Govindasamy L, Agbandje-McKenna M, Srivastava A.** 2007. A dual role of EGFR protein tyrosine kinase signaling in ubiquitination of AAV2 capsids and viral second-strand DNA synthesis. Mol. Ther. **15:**1323–1330.
28. **Zhong L, Li B, Jayandharan G, Mah CS, Govindasamy L, Agbandje-McKenna M, Herzog RW, Weigel-Van Aken KA, Hobbs JA, Zolotukhin S, Muzyczka N, Srivastava A.** 2008. Tyrosine-phosphorylation of AAV2 vectors and its consequences on viral intracellular trafficking and transgene expression. Virology **381:**194–202.
29. **Bogyo M, Wang EW.** 2002. Proteasome inhibitors: complex tools for a complex enzyme. Curr. Top. Microbiol. Immunol. **268:**185–208.
30. **Arastu-Kapur S, Anderl JL, Kraus M, Parlati F, Shenk KD, Lee SJ, Muchamuel T, Bennett MK, Driessen C, Ball AJ, Kirk CJ.** 2011. Nonproteasomal targets of the proteasome inhibitors bortezomib and carfilzomib: a link to clinical adverse events. Clin. Cancer Res. **17:**2734–2743.
31. **Puente XS, Sanchez LM, Overall CM, Lopez-Otin C.** 2003. Human and mouse proteases: a comparative genomic approach. Nat. Rev. Genet. **4:**544–558.
32. **Groll M, Kim KB, Kairies N, Huber R, Crews CM.** 2000. Crystal structure of epoxomicin:20s proteasome reveals a molecular basis for selectivity of alpha,beta-epoxyketone proteasome inhibitors. J. Am. Chem. Soc. **122:**1237–1238.
33. **Sin N, Kim KB, Elofsson M, Meng L, Auth H, Kwok BH, Crews CM.** 1999. Total synthesis of the potent proteasome inhibitor epoxomicin: a useful tool for understanding proteasome biology. Bioorg. Med. Chem. Lett. **9:**2283–2288.

34. **Demo SD, Kirk CJ, Aujay MA, Buchholz TJ, Dajee M, Ho MN, Jiang J, Laidig GJ, Lewis ER, Parlati F, Shenk KD, Smyth MS, Sun CM, Vallone MK, Woo TM, Molineaux CJ, Bennett MK.** 2007. Antitumor activity of PR-171, a novel irreversible inhibitor of the proteasome. Cancer Res. **67:** 6383–6391.
35. **Grieger JC, Choi VW, Samulski RJ.** 2006. Production and characterization of adeno-associated viral vectors. Nat. Protoc. **1:**1412–1428.
36. **Adams J, Palombella VJ, Sausville EA, Johnson J, Destree A, Lazarus DD, Maas J, Pien CS, Prakash S, Elliott PJ.** 1999. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res. **59:**2615–2622.
37. **Kuhn DJ, Chen Q, Voorhees PM, Strader JS, Shenk KD, Sun CM, Demo SD, Bennett MK, van Leeuwen FW, Chanan-Khan AA, Orlowski RZ.** 2007. Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin-proteasome pathway, against preclinical models of multiple myeloma. Blood **110:**3281–3290.
38. **Tsubuki S, Saito Y, Tomioka M, Ito H, Kawashima S.** 1996. Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine. J. Biochem. **119:**572–576.
39. **Anwar A, Siegel D, Kepa JK, Ross D.** 2002. Interaction of the molecular chaperone Hsp70 with human NAD(P)H:quinone oxidoreductase 1. J. Biol. Chem. **277:**14060–14067.
40. **Akache B, Grimm D, Shen X, Fuess S, Yant SR, Glazer DS, Park J, Kay MA.** 2007. A two-hybrid screen identifies cathepsins B and L as uncoating factors for adeno-associated virus 2 and 8. Mol. Ther. **15:**330–339.
41. **Johnson JS, Gentzsch M, Zhang L, Ribeiro CM, Kantor B, Kafri T, Pickles RJ, Samulski RJ.** 2011. AAV exploits subcellular stress associated with inflammation, endoplasmic reticulum expansion, and misfolded proteins in models of cystic fibrosis. PLoS Pathog. **7:**e1002053. doi:10.1371/journal.ppat.1002053.
42. **Qiu M, Chen Y, Cheng L, Chu Y, Song H-Y, Wu Z-W.** 2013. Pyrrolidine dithiocarbamate inhibits herpes simplex virus 1 and 2 replication, and its activity may be mediated through dysregulation of the ubiquitin-proteasome system. J. Virol. **87:**8675–8686.
43. **Wang Y, Li XL, Yu YS, Tang ZH, Zang GQ.** 2013. Inhibition of hepatitis B virus production in vitro by proteasome inhibitor MG132. Hepatogastroenterology **60:**837–841.
44. **Gentile M, Recchia AG, Mazzone C, Lucia E, Vigna E, Morabito F.** 2013. Perspectives in the treatment of multiple myeloma. Expert Opin. Biol. Ther. **13:**S1–S22.