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Journal of Virology

# Identification and Validation of Small Molecules That Enhance Recombinant Adeno-associated Virus Transduction following High-Throughput Screens

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**ABSTRACT**

**While the recent success of adeno-associated virus (AAV)-mediated gene therapy in clinical trials is promising, challenges still face the widespread applicability of recombinant AAV(rAAV). A major goal is to enhance the transduction efficiency of vectors in order to achieve therapeutic levels of gene expression at a vector dose that is below the immunological response threshold. In an attempt to identify novel compounds that enhance rAAV transduction, we performed two high-throughput screens comprising 2,396 compounds. We identified 13 compounds that were capable of enhancing transduction, of which 12 demonstrated vector-specific effects and 1 could also enhance vector-independent transgene expression. Many of these compounds had similar properties and could be categorized into five groups: epipodophyllotoxins (group 1), inducers of DNA damage (group 2), effectors of epigenetic modification (group 3), anthracyclines (group 4), and proteasome inhibitors (group 5). We optimized dosing for the identified compounds in several immortalized human cell lines as well as normal diploid cells. We found that the group 1 epipodophyllotoxins (teniposide and etoposide) consistently produced the greatest transduction enhancement. We also explored transduction enhancement among single-stranded, self-complementary, and fragment vectors and found that the compounds could impact fragmented rAAV2 transduction to an even greater extent than single-stranded vectors. *In vivo* analysis of rAAV2 and all of the clinically relevant compounds revealed that, consistent with our *in vitro* results, teniposide exhibited the greatest level of transduction enhancement. Finally, we explored the capability of teniposide to enhance transduction of fragment vectors *in vivo* using an AAV8 capsid that is known to exhibit robust liver tropism. Consistent with our *in vitro* results, teniposide coadministration greatly enhanced fragmented rAAV8 transduction at 48 h and 8 days. This study provides a foundation based on the rAAV small-molecule screen methodology, which is ideally used for more-diverse libraries of compounds that can be tested for potentiating rAAV transduction.**

**IMPORTANCE**

**This study seeks to enhance the capability of adeno-associated viral vectors for therapeutic gene delivery applicable to the treatment of diverse diseases. To do this, a comprehensive panel of FDA-approved drugs were tested in human cells and in animal models to determine if they increased adeno-associated virus gene delivery. The results demonstrate that particular groups of drugs enhance adeno-associated virus gene delivery by unknown mechanisms. In particular, the enhancement of gene delivery was approximately 50 to 100 times better with than without teniposide, a compound that is also used as chemotherapy for cancer. Collectively, these results highlight the potential for FDA-approved drug enhancement of adeno-associated virus gene therapy, which could result in safe and effective treatments for diverse acquired or genetic diseases.**

Adeno-associated viral (AAV) vectors have emerged to be one of the most promising types of vectors for gene therapy. Indeed, recent and ongoing clinical trials have reported improvements in patients with hemophilia B (1), Parkinson's disease (2, 3), Leber congenital amaurosis (4–7), and Canavan disease (8). Such clinical successes have led to the approval of recombinant AAV (rAAV)-mediated gene therapy in the European Union for the treatment of lipoprotein lipase deficiency (LPLD) (9). Enthusiasm for using rAAV vectors stems from the unique properties of the virus itself. As naturally occurring AAV requires a helper virus such as adenovirus or herpes simplex virus in order to carry out a productive infection, AAV on its own is not known to cause disease in humans. AAV vectors are comprised of transgenic DNA (<5 kb) with the only viral sequence being the 145-nucleotide inverted terminal repeats (ITRs). Furthermore, naturally occurring serotypes and engineered capsids have been shown to display diverse tissue tropism, as well as the ability to infect both dividing and nondividing cells (for a review, see reference 10). From a vector perspective, the use of AAV for gene therapy applications is limited only by the size of the vector, which consists of a transgene of approximately 4.7 kb (11). However, even the size limitations of rAAV vectors are being challenged with the development of trans-splicing and fragment vector (fAAV) technology; that is, transgene

Received 9 December 2015 Accepted 20 April 2016
Accepted manuscript posted online 4 May 2016
Citation Nicolson SC, Li C, Hirsch ML, Setola V, Samulski RJ. 2016. Identification and validation of small molecules that enhance recombinant adeno-associated virus transduction following high-throughput screens. J Virol 90:7019–7031. doi:10.1128/JVI.02953-15.
Editor: G. McFadden, University of Florida
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cassettes that rely on cellular recombination pathways to restore a full-length, large genome upon delivery by multiple rAAV vectors (12–19).

Despite its remarkable safety profile, gene therapy using AAV vectors has had limited success in applications requiring systemic delivery, namely, to nonimmunoprivileged sites (as opposed to direct injection into various tissues or to immunoprivileged regions). Results from clinical trials utilizing either rAAV2 or rAAV8 to deliver human factor IX to hemophilia B patients have suggested that capsid-specific cytotoxic T lymphocytes (CTLs) might have eliminated the majority of transduced cells, thus impeding successful gene expression (1, 20). In these dose escalation trials, therapeutic protein levels were achieved at the highest vector doses administered; however, these levels were transient and their decline corresponded with a rise in liver transaminases, suggestive of transduced cell death. There was no rise in liver enzymes detected at the lower doses tested, suggesting that gene transfer to achieve protein levels that would be deemed therapeutic is limited by the amount of capsid that can be delivered to the host (21). It has thus been postulated that if transduction efficiency could be improved (including both gene transfer and expression), fewer vectors would be required to achieve a given level of gene expression, and thus a clinically relevant therapeutic protein level could be obtained without eliciting a host immune response (22).

Several strategies have been employed in order to enhance transduction efficiency. Both rational mutagenesis and library-based approaches have been designed to create capsids that have increased transduction efficiency for particular tissue types as well as decreased tropism for nontarget organs such as the liver (23–28). Barriers within the intracellular trafficking pathway have also been circumvented based on rationally engineered capsids (29, 30). Improvements to transgene design, including self-complementary rAAV and tissue-specific promoters, ensure faster and more-robust onset of gene expression in target tissues (31–34). Finally, enhancing transduction efficiency through the use of pharmacological agents has been explored. Previous work has shown that topoisomerase inhibitors and anthracyclines can enhance transduction both *in vitro* and *in vivo* (35–42). Proteasome inhibitors, particularly the FDA-approved bortezomib (Velcade), have been shown to enhance transduction *in vitro* as well as in both small- and large-animal models (39, 40, 43). Collectively, these strategies have been important in reaching the level of transduction required for therapeutic benefit without eliciting a host immune response; however, efficient transduction in a wide variety of clinical applications is still a major goal that is actively being pursued.

In this study, we performed a high-throughput, small-molecule screen with the purpose of identifying additional compounds that enhance rAAV2 transduction. Validation of this approach was confirmed by the transduction augmentation of the previously identified topoisomerase II inhibitors, anthracyclines, and proteasome inhibitors in our small-molecule screen. Additionally, we identified several novel compounds and further characterized those with reported functions based on existing literature. Collectively, these transduction-enhancing compounds could be grouped into 5 categories: epipodophyllotoxin topoisomerase II inhibitors (group 1), DNA damage inducers (group 2), effectors of epigenetic modification (group 3), anthracyclines (group 4), and proteasome inhibitors (group 5). We also identified a single agent that enhanced rAAV2 transduction through an unrelated mechanism. The ability of these compounds to enhance transduction was confirmed *in vitro* using several human cell lines, including a normal diploid cell line, NHF-1. Some of these compounds were shown to be effective in enhancing self-complementary rAAV2 (scAAV2) but to a lesser extent than single-stranded rAAV2. Interestingly, the compounds exerted the greatest degree of enhancement on fAAV2, restoring transduction to levels equivalent to or surpassing that of single-stranded rAAV2. We further examined the activity of compounds identified in our screen that are currently approved for clinical use *in vivo* and found that, consistent with our *in vitro* results, one such compound—teniposide—exhibited the greatest level of transduction enhancement. Given the impact of teniposide on fAAV2, we explored its capability to enhance transduction of fragment vectors *in vivo* using an AAV8 capsid that is known to exhibit robust liver tropism. Consistent with our *in vitro* results using fAAV2, teniposide coadministration enhanced fragmented rAAV8 transduction both at 48 h and through the duration of the experiment (8 days). Our results demonstrate a simple, effective method of discovering compounds that enhance rAAV2 transduction. We anticipate that this approach can be applied to vectors derived from other serotypes and in other cell lines. Furthermore, our design provides a foundation to investigate the plethora of commercially available compound libraries that span the small-molecule and FDA-approved drug milieu.

## MATERIALS AND METHODS

**Cell culture.** HeLa cells, U87 cells, and normal human fibroblasts (NHF1s) were grown in Dulbecco's modified Eagle medium that was supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 g/ml streptomycin (complete DMEM). HepG2 cells were grown in RPMI 1640 medium, supplemented as described above. All cell lines were maintained at 37°C and 5% $CO_2$.

**Virus production.** Virus was produced in HEK-293 cells as previously described (44). Briefly, using polyethylenimine (PEI) Max (Polysciences), cells were triple transfected with a Rep and Cap plasmid (pXR2 or pXR8), an inverted terminal repeat-flanked transgene plasmid (single-stranded pTR-CBA-Luciferase, self-complementary pTR-CMV-gaussia-Luciferase, or oversized pTR-CBA-Luciferase, where CBA is chicken-beta actin and CMV is cytomegalovirus), and the pXX6-80 helper plasmid. Between 48 and 72 h posttransfection, cells were harvested and virus was purified by cesium chloride gradient density centrifugation overnight at 55,000 rpm. Fractions that contained peak virus titers were dialyzed in dialysis buffer (1× phosphate-buffered saline [PBS], 0.5% sorbitol, 0.5 mM calcium chloride, and 1 mM magnesium chloride). Titers were calculated by quantitative PCR (qPCR) according to established procedures (16) by using a LightCycler 480 instrument with Sybr green PCR master mix. Conditions used for the reaction were as follows: 1 cycle at 95°C for 10 min; 45 cycles at 95°C for 10 s, 62°C for 10 s, and 72°C for 10 s; and 1 cycle at 95°C for 30 s, 65°C for 1 min, and 99°C for acquisition.

**Compound screen, 384-well format.** HeLa cells were plated at least 18 h prior to compound treatment and infection at a density of $8 \times 10^3$ cells/well. Compounds were prepared in complete DMEM so that delivery would yield a final concentration of 1 μM. Compounds were added directly to wells. Two hours posttreatment, rAAV2-CBA-Luciferase was administered at a dose of 1,000 vector genomes (vg)/cell. Cells were harvested 24 h postransduction by medium removal followed by incubation with passive lysis buffer (Promega) for 15 min. Luciferase activity was measured in accordance with the manufacturer's instructions (Promega). Luciferase activity was measured either a PerkinElmer 1450 MicroBeta TriLux LSC and luminescence counter or a PerkinElmer 2450 MicroBeta[2] microplate counter. Compounds that enhanced transduction >2-fold over dimethyl sulfoxide (DMSO) treatment were considered hits for fur-

**TABLE 1** Compounds characterized in this study[a]

| Group no. | Compound name | NSC ID[b] | Clinical use | Group | Known mechanism of action |
|---|---|---|---|---|---|
| 1 | Teniposide | 122819 | Chemotherapy | Podophyllotoxin | Topoisomerase II inhibition |
| | Etoposide | 141540 | Chemotherapy | | |
| 2 | Bleomycin | 125066 | Chemotherapy, plantar warts | Glycopeptide antibiotic | DNA damage |
| | Parthenin | 85239 | | Sesquiterpene lactone | |
| | RH-1 | 697726 | Phase I, solid malignancies | Diaziridinylbenzoqui none | |
| 3 | Vorinostat | 701852 | Chemotherapy | HDAC inhibitor | HDAC inhibition |
| | Nanaomycin A | 267461 | | DNMT3B inhibitor | DNMT3B inhibition |
| 4 | Menogaril | 269148 | | Anthracycline | DNA intercalation, topoisomerase II inhibition, polymerase inhibition, free radical damage to DNA |
| | Pyrromycin | 267229 | | | |
| | Daunorubicin | 82151 | Chemotherapy | | |
| 5 | Bortezomib | 681239 | Chemotherapy | Dipeptide | Proteasome inhibition |
| | Physalin B | 287088 | | Physalin | |
| | Siomycin | 285116 | | Thiazole antibiotic | |
| | Tetrocarcin A | 333856 | | Microbial metabolite | BCL-2 inhibitor |

[a] Compounds that emerged as hits were selected based on known function in cells or clinical utility.
[b] NSC ID, National Service Center identifying number.

ther study. Cell viability was measured using the CellTiterGlo luminescent cell viability assay (Promega).

**Secondary screen, 96-well format.** HeLa cells were plated at least 18 h prior to compound treatment and infection at a density of $2 \times 10^4$ cells/well. Compounds were prepared in complete DMEM at a concentration of 10 μM. Medium was replaced with medium containing each compound. Two hours posttreatment, rAAV2-CBA-Luciferase was administered at a dose of 500 vg/cell. Cells were harvested 24 h postinfection via incubation with passive lysis buffer (Promega) for 15 min. Luciferase activity was measured in accordance with the manufacturer's instructions (Promega). Luciferase activity was measured with a Wallac 1420 Victor3 plate reader. Compounds that enhanced transduction >5-fold over DMSO treatment were considered hits for further study.

**Transduction assays.** Cells were plated at least 18 h prior to infection in 96-well plates at a density of $2 \times 10^4$ cells/well. Compound treatment was performed 2 h prior to infection. Compounds were either provided by the Drug Testing Program or were commercially available (teniposide from Sigma; nanaomycin from Apex Bio; daunorubicin and vorinostat from LC Laboratories). Cells were infected with purified rAAV2-CBA_Luc at 500 vector genomes/cell. Cells were harvested 24 h postinfection, and luciferase activity was determined as described above. For scAAV, cells were treated as described above. At 24 h postinfection, 20 μl of medium was transferred to a black 96-well plate. The luciferase assay was performed using coelenterazine (Nanolight) as the reagent. Briefly, the coelenterazine was resuspended to 10 mg/ml in methanol. To make the working solution, the concentrated stock was dissolved in Tris-EDTA (TE) buffer containing NaCl (0.6 M) at a 1:200 dilution. The working solution was added to the wells at a 1:1 ratio of medium to coelenterazine, and luciferase activity was recorded.

**Transfection assays.** Two million HeLa cells were plated at least 18 h prior to transfection in a 10-cm plate. Cells were transfected with 1 μg pTR-CBA-Luc using PEI Max. Twenty-four hours posttransfection, cells were plated in a 96-well plate at a density of $2 \times 10^4$ cells/well. At 48 h posttransfection, cells were treated with the indicated drugs. At 72 h posttransfection, cells were harvested and luciferase activity was measured.

**Animal studies.** Housing and handling of BALB/c mice used in the study were carried out in compliance with National Institutes of Health guidelines and approved by the IACUC at the University of North Carolina Chapel Hill. All drugs and rAAV-Luc were coadministered through the intravenous route (tail vein) in a total volume of 200 μl (normalized with l× PBS). For toxicity analysis, 24 h postadministration blood was collected and serum was assessed for blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatine kinase (CK). Bioluminescence of Luc expression was visualized by using a Xenogen IVIS Lumina imaging system (PerkinElmer) following intraperitoneal injection of luciferin substrate (120 μl; Nanolight). Image acquisition and analysis were carried out by using Living Image software.

## RESULTS

**Primary and secondary screens.** The overall goal of our study was to identify and characterize small molecules that enhanced rAAV transduction *in vitro* and, if the small molecule was FDA approved at the time, *in vivo*. Two screens were performed to identify such compounds. The first was a stringent screen using a 1 μM final compound concentration to identify compounds capable of potentiating rAAV2 transduction at low concentrations. In each plate, column 1, row 1 contained no virus and no compounds, column 2, row 2 contained rAAV2 but no compounds. Column 24, row 4 contained 1 μM MG132 as a positive control. Column 23, row 3 contained rAAV2 with only DMSO (vehicle control). All compounds were administered in duplicate. Finally, each plate contained two sets of the given controls and compounds, whereby rows 1 to 8 (top, gray) were assayed for transduction activity and rows 9 to 16 (bottom, white) were assayed for viability using the CellTiterGlo system. As the vast majority of AAV biology has been discovered using AAV2 or rAAV2 in HeLa cells, our primary and secondary screens were performed using these parameters. Owing to its wide range in output and linear relationship to vector dose, the CBA-luciferase transgene, and thus luciferase activity, was chosen as the reporter for transduction efficiency. A secondary screen utilized a high (10 μM) final compound concentration in tandem with employing rAAV2 to HeLa cells at 500 vg/cell. The format of this screen utilized DMSO added to all wells in column 1, row 1 as a vehicle control, while two wells in column 12, row 2 contained MG132 as a positive control. Cell viability was not assessed at this stage. A combined list of the compounds that were pursued further is provided in Table 1.

In the primary screen, an initial "hit" was defined as a compound that enhanced transduction at least 2-fold, while in the secondary screen, an initial "hit" was defined as a compound that enhanced transduction at least 4-fold. From the hits found in both screens (a total of 72), we chose to further study compounds that had known functions based on existing literature. However, it is noteworthy to mention that several compounds with as-yet-unknown function were identified as hits, some enhancing transduction 5- to 10-fold. These compounds warrant validation in further studies, but further characterization was deferred in order to better understand the compounds with reported mechanisms of action. Of these hits, compounds could be further categorized into groups based on their known cellular mechanisms of action (Table 1). Group 1 compounds included the epipodophyllotoxins teniposide and etoposide, which have been shown to inhibit topoisomerase II activity and cause DNA damage. Of these, etoposide has been previously identified as having rAAV2 transduction-potentiating activity, likely through mechanisms related to DNA damage repair proteins induced by etoposide-mediated inhibition of topoisomerase II (38). Group 2 compounds comprised other small molecules that are known to cause DNA damage, although not necessarily through topoisomerase II inhibition. These included the glycopeptide antibiotic bleomycin, which is known to cause DNA double-strand breaks (45), the sesquiterpene lactone parthenin, which has been shown to induce chromatid breaks (46), and the diaziridinylbenzoquinone RH1, a DNA cross-linker (47).

Group 3 compounds, nanaomycin A and vorinostat, are characterized by their ability to facilitate epigenetic modifications of DNA. While the quinine antibiotic nanaomycin A has been shown to generate oxygen free radicals and cause DNA damage (48, 49), it has also recently been identified as an inhibitor of DNA methyltransferase 3B (DNMT3B) (50), thereby inhibiting epigenetic repression of gene expression. Vorinostat (suberanilohydroxamic acid [SAHA]) belongs to the class of histone deacetylase inhibitors, which function in cells to cause an accumulation of acetylated histones and transcription factors, which in general increases gene expression (51, 52). Group 4 compounds consisted of anthracyclines, which are compounds that have been shown to exert a variety of effects, including inhibition of topoisomerase and proteasomal activity. These compounds included menogaril, pyrromycin, and the chemotherapeutic daunorubicin. A related anthracycline, doxorubicin, has been characterized for its enhancement of rAAV transduction (39, 40); therefore, it is likely that these other anthracyclines potentiate rAAV transduction through similar mechanisms.

We also identified several nonanthracycline compounds that have been shown to have proteasome-inhibiting activity (group 5). These included bortezomib, physalin B, and siomycin. Bortezomib, an FDA-approved proteasome-inhibiting agent, has been explored for rAAV applications in a variety of cell lines and animal models and using different serotypes (43, 53–56). Thus, the identification of bortezomib within the screen inadvertently served as an internal control in validating our experimental setup. In addition to its proteasome-inhibiting properties (57), physalin B has also been shown to have anti-inflammatory and other immunomodulatory activity (58). The proteasome-inhibiting activity of siomycin has been correlated with its role as an inhibitor of the transcription factor forkhead box M1 (FOXM1) (59–61).

Finally, we identified one compound, tetrocarcin A, that could not be categorized into any of groups 1 to 4 but did show modest levels of rAAV2 transduction enhancement. Tetrocarcin A has been shown to inhibit the antiapoptotic function of Bcl-2 (62) and induce endoplasmic reticulum (ER) stress (63), the latter condition of which we previously demonstrated to increase transduction (64).

In addition, our screen yielded several compounds that are known to interfere with microtubule dynamics, including colchicine, vincristine, and vinblastine, among others; however, these were excluded from further study as it has already been demonstrated that these microtubule inhibitors inhibit the cell cycle at low concentrations (65), which is likely the mechanism behind their transduction enhancement. In fact, at higher concentrations, these drugs have been shown to inhibit viral trafficking to the nucleus as well as transduction (66, 67).

**Dose optimization of compounds in HeLa cells.** In order to further characterize the active compounds from our screen, we wanted to determine an optimal concentration that would result in the greatest enhancement of transduction without causing overt toxicity. To avoid potential side effects caused by DMSO, the final concentration of compounds was limited to 100 μM for high-dose applications. Based on these concentration boundaries, it is possible that compounds that both were well tolerated by cells and substantially enhanced transduction at higher concentrations (such as etoposide and bleomycin) may be capable of enhancing transduction at an even greater level than what was determined under our experimental conditions. For some compounds, the therapeutic window was quite small in that there was a very sharp decrease in cell viability as the concentration of the compound was increased. A notable example was menogaril, which at 5 μM showed a $>$10-fold enhancement and approximately 100% viability but at 10 μM resulted in an approximately 50% reduction in viability, which corresponded to a reduction in transduction enhancement as well (data not shown). The epipodophyllins teniposide and etoposide, as well as the DNA-damaging agent bleomycin, were the strongest augmenters in this assay, each increasing transduction over 20-fold. The proteasome inhibitors bortezomib and physalin B also showed enhancement over 10-fold.

**Effects of compounds on vector transduction versus general gene expression.** Given the identified compounds' impressive utility across multiple cell lines, it was important to investigate whether the observed increase in transgene expression was due to a mechanism related specifically to rAAV vectors. To this end, HeLa cells were transfected with pTR-CBA-Luc, the transgene plasmid used to generate the above-described single-stranded vector constructs, and assayed for enhancement of luciferase activity following compound administration (Fig. 1). Most compounds elicited a negligible change in luciferase activity. For example, physalin B induced an approximately 2-fold increase in plasmid luciferase expression but a $>$10-fold increase in transduction, Some of the compounds even elicited a decrease in gene expression when tested with the plasmid vector cassette. The only exception was the histone deacetylase (HDAC) inhibitor vorinostat, which increased luciferase expression of the transfected plasmid to an extent similar to that seen with incoming vectors. It is noted that increased plasmid-borne transgene expression in this case does not simply imply that a similar mechanism of enhancement occurs with AAV vectors. However, in attempts to focus on drug enhancement of AAV vectors specifically, vorinostat was excluded from further analysis.

**Characterization of compound activity in human cell lines.** Given the ability of the identified compounds to enhance the

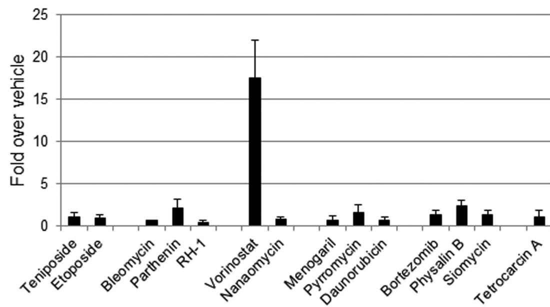


**FIG 1** Effects of compounds on plasmid gene expression. HeLa cells were transfected with pCBA-Luc and treated with the compounds identified in our screen. Transduction was assessed 24 h after compound treatment. Of the hits, vorinostat appears to be the only compound that enhances plasmid gene expression.

transduction of rAAV2 in HeLa cells (Fig. 2A), we further explored the utility of these compounds in different cell types to better understand their potential universal utility. The first such cell line examined was U87, a human glioblastoma cell line that is amenable to rAAV2 transduction (Fig. 2B). Overall, the fold enhancement of each of the compounds was very similar to what was observed in HeLa cells, with a few exceptions. While teniposide enhanced transduction approximately 40-fold in HeLa cells, enhancement was seen at 20-fold in U87 cells. Interestingly, while bortezomib enhanced transduction by approximately 15-fold in HeLa cells, enhancement was only approximately 3-fold in U87 cells. However, the level of enhancement obtained with by another compound with proteasome-inhibiting activity, physalin B, remained similar in both cell lines (12-fold in HeLa cells and 10.5-fold in U87 cells). We next evaluated these compounds in HepG2 cells, a hepatocellular carcinoma cell line, as the liver is the site of transduction for systemically delivered rAAV2 (Fig. 2C). For treatment with pyrromycin, nanaomycin A, physalin B, bortezomib, and tetrocarcin A, transduction enhancement was observed at approximately the same magnitude as that seen with HeLa cells. Treatment with the epipodophyllotoxins, anthracyclines, siomycin, and RH-1 resulted in enhancements 4- to 12-fold greater than that observed in HeLa cells. Notably, menogaril, which enhanced rAAV2 transduction in HeLa cells approximately 12-fold, enhanced transduction in HepG2 cells 138-fold. Finally, our compound collection was applied to NHF1 cells (a kind gift from William Kaufman), a diploid cell line derived from neonatal foreskin and immortalized by the expression of telomerase reverse transcriptase (hTERT) that displays contact inhibition and stationary growth once confluent (68) (Fig. 2D). Again, treatment with the epipodophyllotoxins and anthracyclines (especially menogaril) significantly enhanced transduction at magnitudes much greater than what was observed in HeLa cells. Treatment of NHF-1 cells with bleomycin also resulted in impressive transduction enhancement (approximately 100-fold). These results suggest that in general, the group 1 epipodophyllotoxins, causing DNA damage from topoisomerase II inhibition, consistently yielded the greatest enhancement in transduction. Proteasome inhibition, particularly by bortezomib, was more variable and cell type dependent. It is possible that the enhancing ability of these

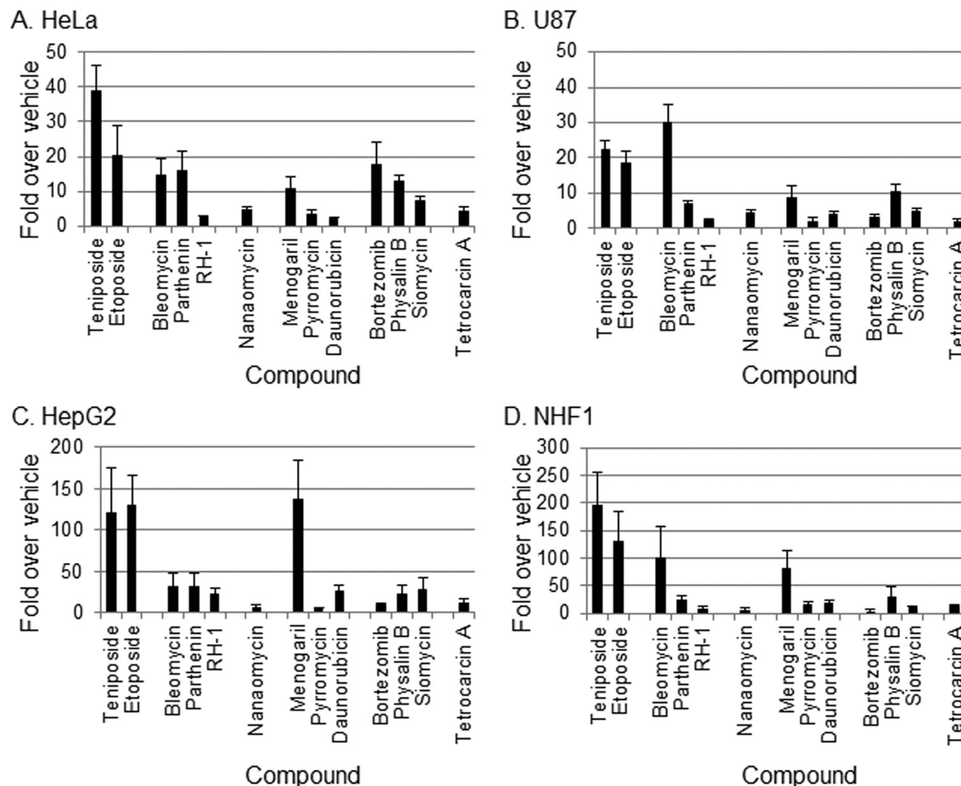


**FIG 2** Effects of compounds on human cell lines. Cells were treated with the identified compounds or dose-appropriate DMSO and later rAAV2_CBA-Luc. Transduction was assessed 24 h after drug treatment. (A) HeLa cells; (B) U87 cells; (C) HepG2 cells; (D) NHF1 cells.

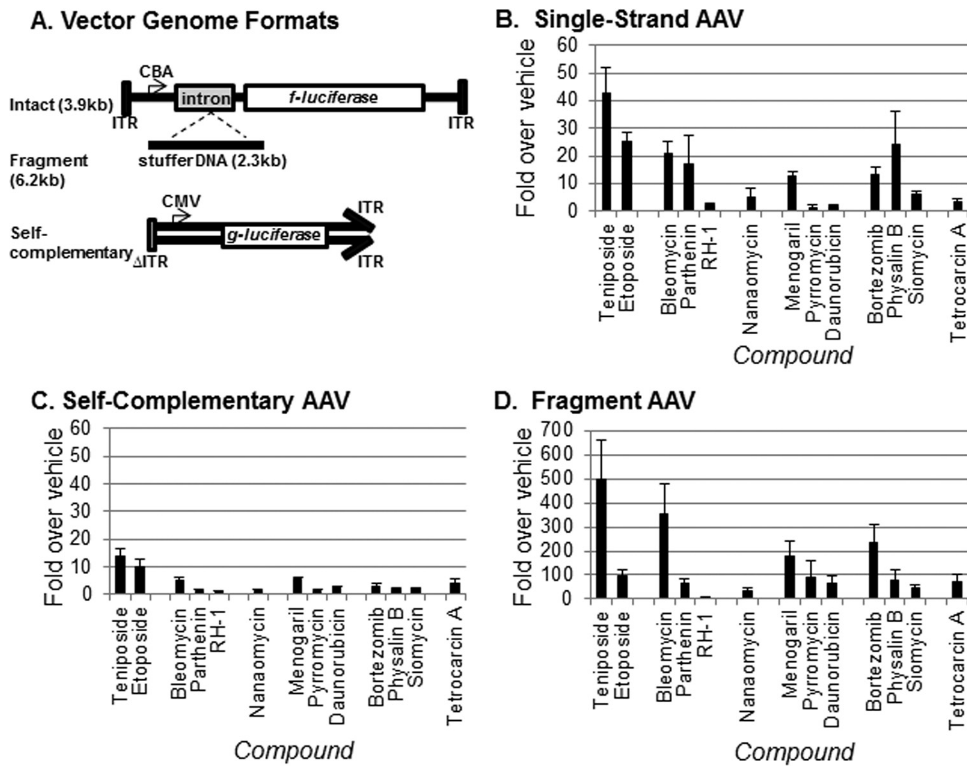


**FIG 3** Effects of identified compounds on vectors with different forms of transgenes. (A) Schematic of each of the transgene cassettes utilized in this study. Top, single-stranded, intact; middle, self-complementary; bottom, fragment. HeLa cells were treated with each of the identified compounds and single-stranded rAAV2 (B), scAAV2 (C), or fAAV2 (D) or dose-appropriate DMSO. Luciferase activity was measured 24 h following compound treatment.

compounds depends on the abundance of their actionable targets within cells. Thus, these results highlight the differences in response to pharmacological modulation in different cell types, which may influence the type of agent chosen to complement rAAV-mediated gene therapy in a given target tissue.

**Characterization of transduction-specific compound activity with vectors comprised of differentially formed transgenes.** Recent and emerging gene therapy applications have utilized transgenes that differ in form from the prototypical single-stranded AAV genome. For example, self-complementary vectors contain a transgene whereby a third inverted terminal repeat (ITR) in the middle of the transgene is mutated to eliminate the Rep nicking stem (33). This yields a double-stranded gene product, thus eliminating the rate-limiting step of second-strand synthesis and providing a faster, more robust onset of expression (33, 69). Other emerging vectors designed to deliver large transgenes are derived from the forced packaging of oversized genomes into the viral capsid (13, 15, 16, 18, 19, 70). It is thought that the packaged DNA becomes truncated at the 5′ end once AAV has reached its packaging limit, but delivery of these transgene "fragments" still results in expression of the intact transgene through a reannealing process modulated by the homologous recombination protein Rad51C (16). However, the transduction efficiency of fAAV is impaired compared to that of rAAV or scAAV.

We therefore wanted to test the capabilities of the screen-identified compounds in enhancing the transduction of scAAV and fAAV in HeLa cells. For scAAV, a vector carrying a cytomegalovirus promoter-driven *Gaussia* luciferase transgene (CMV-gLuc) was utilized. For fAAV, a previously described vector carrying the same promoter and transgene as the single-stranded vector (CBA-Luc), but with an additional 2.3-kb "stuffer sequence" inserted into an intronic region upstream of the luciferase (16), was utilized. A schematic of each of these vector transgenes is provided in Fig. 3A. We applied the same optimized concentration of each compound and tested their ability to enhance scAAV and fAAV transduction at the same vector dose. With the exception of daunorubicin and tetrocarcin A, the compounds still enhanced scAAV transduction, but the magnitude of the effect was significantly reduced (Fig. 3B). This suggests that these compounds may also be acting at the step of second-strand (ss) synthesis. Since we still observed enhancement of scAAV, albeit at a reduced capacity compared to ssAAV, it is likely that these compounds augment steps in transduction either before second-strand synthesis (such as subcellular trafficking, nuclear entry, or uncoating) or in establishing the persistence of gene expression following second-strand synthesis. Impressively, application of the compounds in conjunction with fAAV2 enhanced transduction to a much greater extent than with rAAV2 (Fig. 3C). Notably, treatment with teniposide, bortezomib, or bleomycin enhanced transduction over 250-fold, which was an 8- to 10-fold increase over the enhancement seen in rAAV2. This result is particularly interesting, as each of these drugs produces a different reported effect within cells. In untreated cells, fAAV2 transduction was approximately 21- to 28-fold lower than intact rAAV2, which is in agreement with previously reported results comparing fAAV2 and intact rAAV2 at 5,000 vg/cell (16). Treatment with any of the compounds restored transduction of fAAV2 to levels (i) above untreated intact rAAV2 levels and (ii) within 2- to 10-fold of drug-treated intact rAAV2

levels, depending on which compound is used. Taken together, these results suggest that the identified compounds can enhance the transduction efficiency of vectors with different forms of transgenes and disproportionately benefit fAAV2 transduction in HeLa cells.

***In vivo* analysis of FDA-approved hits.** Owing to the appeal of repurposing FDA-approved drugs to augment rAAV-mediated gene transfer, we performed an *in vivo* comparison of rAAV2 transduction-potentiating capabilities of the hits identified in our screen that are already FDA approved. Therefore, teniposide, etoposide, bleomycin, daunorubicin, and bortezomib were assessed. The doses selected for *in vivo* study were chosen based on conversions from the FDA-approved dosing in humans to the murine equivalent based on body surface area and established $K_m$ factors (71). As teniposide and etoposide are recommended for use over a wide range of doses, they were administered in this study at a conservative dose of 20 mg/kg of body weight (human equivalent dose, (60 mg/m$^2$). Each pharmaceutical was coadministered with $1 \times 10^{11}$ vg rAAV2-CBA-Luc through tail vein injection in age-matched female BALB/c mice. Two vehicle cohorts were included in the study. We evaluated toxicity by measuring the levels of blood urea nitrogen (BUN), aspartate transaminase (AST), alanine transaminase (ALT), and creatine kinase (CK). These levels appeared within normal ranges and were comparable to those in the vehicle-treated mice. Average levels were similar for drug-treated and vehicle-treated cohorts (data not shown).

Due to the impressive enhancement that we observed *in vitro* at 24 h postransduction, an early time point of gene expression was measured to determine whether the pharmaceuticals could also provide rapid, high-level gene expression *in vivo*. At 48 h postadministration, all of the selected drugs enhanced transduction *in vivo*, but to various degrees (Fig. 4A). Consistent with what was seen *in vitro*, the epipodophyllotoxins as well as daunorubicin enhanced transduction to the greatest extent. Notably, cotreatment with teniposide enhanced transduction an average of nearly 100-fold (Fig. 4B). While teniposide and etoposide have the same mechanism of action in cells (i.e., inhibition of the religation of DNA ends through interference of the DNA-topoisomerase complex), teniposide was a more potent augmenter of transduction at this time point at the given dose. Upon assessment of transduction 8 days postadministration, enhanced transduction was noted among all of the drug-treated cohorts (Fig. 4A). Teniposide coadministration yielded the greatest enhancement in transduction at this time point, followed by bortezomib. Daunorubicin cotreatment also yielded impressive transduction enhancement at the 8 day time point. Bleomycin and etoposide cotreatment produced only modest enhancement of transduction efficiency. This result could be reflective of the *in vivo* dose limitations in order to reflect clinical levels of each drug. In general, variability among treatment groups, including vehicle-treated mice, was noticed and could have been due to either an immune response against the luciferase transgene in some mice, as has been historically observed, or perhaps an effect of the vehicle (DMSO) on *in vivo* transduction.

Because coadministration of rAAV2 with teniposide showed the most-robust transduction enhancement, and because of the robust enhancement observed for fAAV2, we chose to test the ability of teniposide to enhance fragment rAAV *in vivo* as well. Due to the limited systemic transduction capability of fAAV *in vivo* (16), we chose to test the transduction using an AAV8 capsid, as intact rAAV8 vectors have been shown to exhibit strong liver transduction (1, 44, 72). Due to the difficulty in producing high-titer fAAV8, mice were administered only $5 \times 10^{10}$ vg. At 48 h postransduction, the teniposide-treated cohort exhibited 34-fold-higher levels of luciferase activity than the vehicle-treated cohort. Impressively, this enhancement was even greater at 8 days postransduction, with the teniposide-treated cohort exhibiting 86-fold-higher luciferase activity than the vehicle-treated cohort (Fig. 5B).

Taken together, these results confirmed our observations *in vitro* and corroborated that the greatest transduction augmentation arises from cotreatment with topoisomerase or proteasome inhibitors. Furthermore, teniposide was shown to exhibit a robust effect on fAAV8 transduction.

## DISCUSSION

Recent gene therapy applications using rAAV have been met with both success and challenges that depend on the indication, route of administration, serotype, and vector dose utilized. Indeed, clinical efficacy for one indication, lipoprotein lipase deficiency, has been achieved to the extent that the first-ever gene therapy product, an rAAV1 vector carrying a lipoprotein lipase transgene, is now approved for use in the European Union (9). Results from clinical trials have exposed the current limitations of rAAV-mediated gene therapy, one of which is the lack of ability to achieve robust, long-term therapeutic gene expression without eliciting an immune response induced by high vector doses. Several strategies have been employed to combat this challenge, including capsid and transgene modification as well as pharmaceutical intervention; however, they have been met with varied success and may be limited to a particular serotype or tissue target. For example, the elimination of certain tyrosines on the capsids of AAV2 and AAV6 has allowed for enhanced transduction efficiency and lower rates of proteasomal degradation (29, 30, 73); however, it has been shown that this strategy cannot be applied to rAAV9 and attempts with rAAV8 have produced mixed results (74, 75). Similarly, the proteasome inhibitor bortezomib has been shown to enhance transduction of both rAAV2 and rAAV8 vectors, resulting in increased transgene expression in both small- and large-animal models (43, 54, 55); however, a recent report has shown that this strategy cannot be applied for rAAV9-mediated therapy for cardiac failure (76). Thus, the need for a robust, widely applicable strategy to enhance transduction would be highly beneficial in the translation of rAAV-mediated gene therapy to a broad range of applications. In addition, compounds that enhance rAAV transduction may fall into classes that are tissue preferred; therefore, new compounds will always be of interest to explore.

In this study, we employed a high-throughput small-molecule screen to identify compounds capable of enhancing rAAV2 transduction. Several compounds were identified, including some of novel origin and some that have been previously defined. These compounds were shown to have various activities on vectors comprised of different forms of transgenes, as well as some differential effects in a variety of cell lines. Finally, selected compounds that are currently being used in clinical applications were validated *in vivo*. Overall, the greatest enhancers of transduction were drugs that inhibit topoisomerase II, in particular, the group 1 epipodophyllotoxins and the group 4 anthracyclines. Generalizing from the entire collection of our identified hits, it seems that rAAV can be enhanced by two mechanisms: (i) inhibition of the proteasome and (ii) induction of DNA damage, which corroborates previously published results (38, 39, 69, 77, 78).

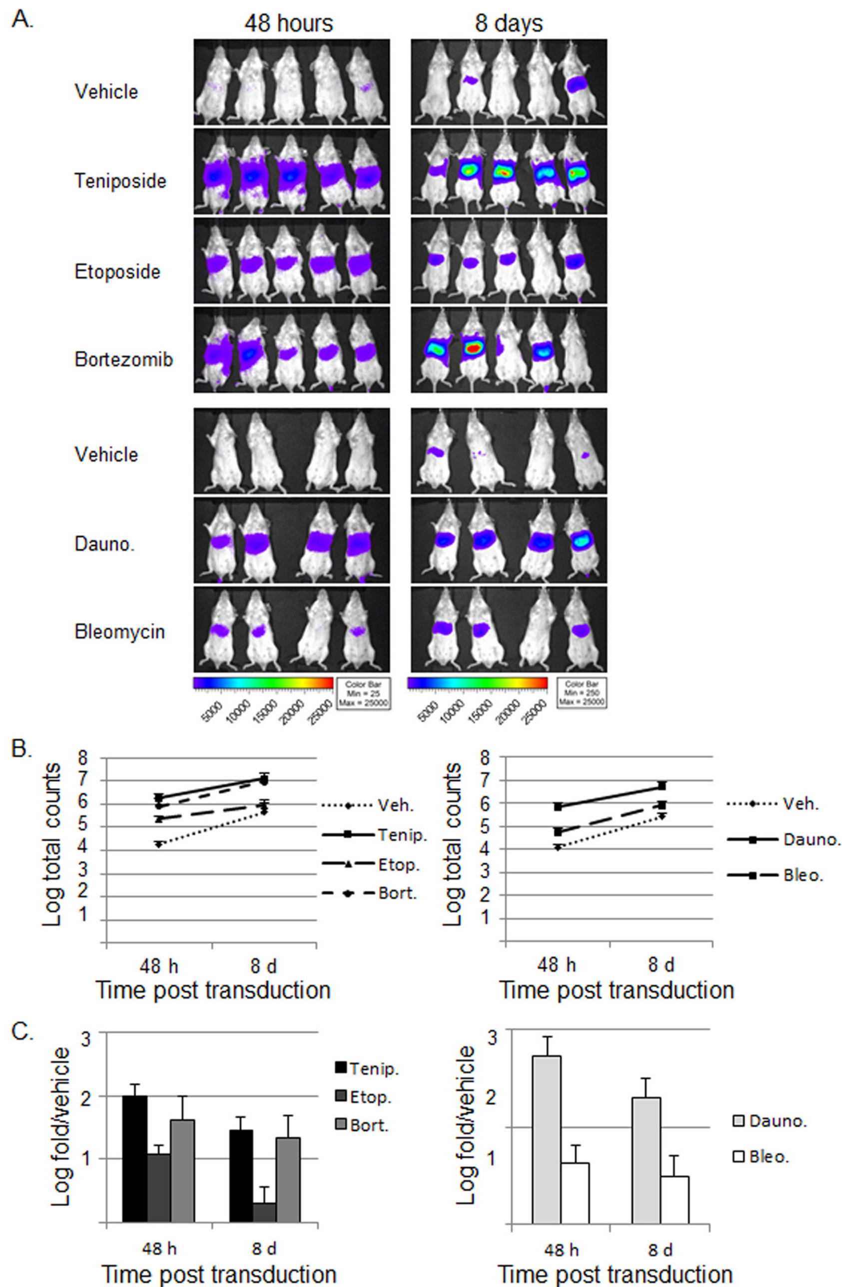


**FIG 4** Analysis of transduction enhancement by compounds *in vivo*. (A) Live bioluminescent imaging of mice that were cotreated with rAAV2_CBA-Luc and each of the clinically relevant compounds, imaged 48 h and 8 days postransduction. (B) Quantification of bioluminescent imaging. (C) Fold difference in luciferase expression over vehicles.

The epipodophyllotoxin and anthracycline topoisomerase II inhibitors form complexes with DNA and topoisomerase II, an enzyme required for the prevention of DNA supercoiling in eukaryotes. The topoisomerase II cycle includes (i) binding and cleavage of duplexed DNA, (ii) passage of a second strand of DNA through the complex, and (iii) religation of the broken DNA ends (79). Both classes of topoisomerase II inhibitors prevent the religation step of this cycle, thus causing both single-stranded and double-stranded DNA damage. While the direct role of topoisomerase II in the mechanism of rAAV transduction enhancement cannot be ruled out, previous research suggests that the DNA damage response, an indirect result of topoisomerase II inhibition, is likely the main contributor to the increase in transduction, either through increased second-strand synthesis or by a currently unknown mechanism (38, 80, 81). Indeed, this screen identified several topoisomerase II-independent DNA-damaging agents, such as bleomycin, parthenin, and RH1, as potentiators of rAAV2 transduction. In fact, bleomycin enhanced transduction to levels similar to those of teniposide and etoposide treatment in U87 cells (Fig. 1A). Several groups have shown that proteins involved in the DNA damage response, including both homologous recombination and nonhomologous end joining, interact with incoming vi-

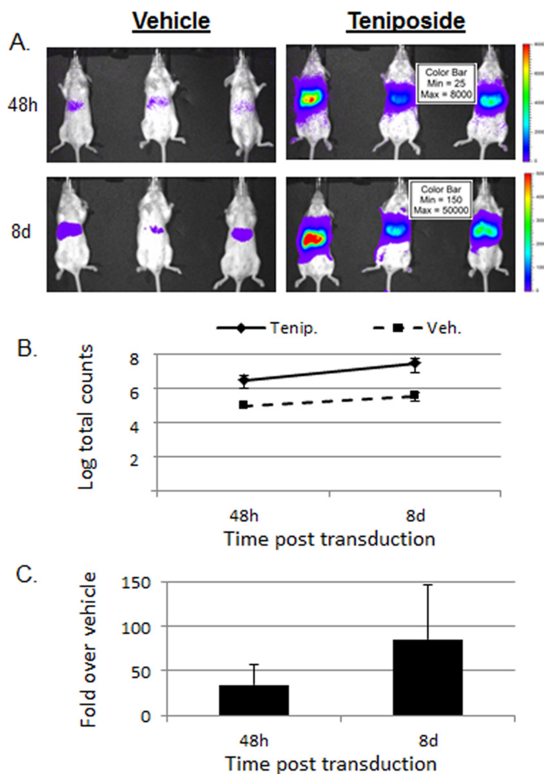


**FIG 5** Analysis of transduction enhancement by fAAV8 with teniposide *in vivo*. (A) Live bioluminescent imaging of mice that were cotreated with fAAV8_Luc and vehicle (top) or teniposide (bottom), imaged 48 h and 8 days postransduction. (B) Quantification of bioluminescent imaging. (C) Fold difference in luciferase expression over vehicles.

ral and vector genomes and have important roles in genome processing. These processes are thought to include the conversion of the single-stranded genome into double-stranded DNA (69, 82), opening of ITR hairpins (83), concatamerization and circularization (78, 84–86), and transgene expression (87). Notably, the Mre11/Rad50/Nbs1 (MRN) complex, important for double-strand break repair, recombination, and telomere maintenance, has been shown to bind to AAV ITRs, negatively affecting rAAV transduction, wild-type AAV (wtAAV) replication, and double-stranded rAAV DNA accumulation (80, 81). It is currently thought that cellular DNA damage, induced by radiation, small molecules, or other means, may serve as a "decoy," recruiting the MRN complex to the sites of damage and away from single-stranded AAV DNA, thus allowing for double-strand conversion. Interestingly, Choi et al. observed that ATM, Mre11, and NBS1 are required for scAAV DNA circularization *in vitro* and ATM and DNA-PK(CS) are required for scAAV DNA circularization *in vivo* (84). Taken together, these results suggest that DNA damage response proteins may have a dual function in rAAV DNA processing and the positive or negative effects may depend on the state of the DNA within the cell.

Proteasome inhibition is thought to be beneficial to rAAV transduction through a mechanism that differs from a DNA damage response. It is believed that inhibition of the proteasome facilitates bulk flow of particles away from degradation pathways, thereby redirecting them to routes that favor transduction (i.e., nuclear translocation) (54, 88, 89). Bortezomib, the only currently FDA-approved proteasome inhibitor, has been used in conjunction with rAAV to enhance transduction in hemophilia A and hemophilia B models (43). Its effects seem to be thus far limited to these applications, as a recent study using rAAV9 to deliver SERCA2a to preserve cardiac function in a rat model demonstrated no additional benefit when bortezomib was coadministered (76). Indeed, our results suggest that the effects of proteasome inhibition by bortezomib are cell type dependent, as bortezomib-mediated enhancement was not observed in U87 and NHF cell lines, and not as robust in HepG2 cells as in HeLa cells. Interestingly, two compounds that have been previously implicated in proteasome inhibition were identified, physalin B and siomycin, which outperformed bortezomib in U87, HepG2, and NHF cell lines. These compounds either may inhibit proteasome function through a mechanism that is different from that of bortezomib or may perform additional functions in cells that are beneficial to rAAV2 transduction.

The level of involvement of DNA damage response proteins and proteasome inhibition seems to differ between ssAAV2, scAAV2, and fAAV2. While the ssAAV2 and fAAV2 used in this study share identical promoters and luciferase genes, the scAAV2 cassette differed in promoter (CMV versus CBA) as well as transgene (*Gaussia* versus firefly luciferase). Therefore, a direct comparison can be made between ssAAV2 vectors and fAAV2 vectors, but considerations must be made when evaluating the performance of the scAAV2 vector. Initial conclusions seem to indicate that, for all of the compounds identified in our screen, transduction enhancement seemed to be less pronounced for scAAV2. This finding is in agreement with the current theory of how DNA damage proteins may be inhibitory for ssAAV DNA, since scAAV DNA does not require second-strand synthesis and would therefore be unaffected by any proteins limiting this type of processing. Since it has been shown that Mre11 and ATM are required for scAAV genome circularization, it will be interesting to evaluate the long-term effects of these compounds on scAAV gene expression in the future. Interestingly, some enhancement of scAAV2 transduction was observed with the cotreatment with the epipodophyllotoxins and anthracyclines, suggesting that these compounds might enhance transduction by mechanisms in addition to facilitating second-strand synthesis.

Perhaps the most striking observation was the dramatic increase in transduction seen for fAAV2 treated with the compounds identified in this screen. Notably, each compound boosted fAAV2 transduction to levels greater than in ssAAV2 treated with vehicle alone. Previous studies have shown that fAAV2 transduction relies on the annealing of sections of vector DNA that comprise the entire oversized transgene cassette (16). We previously noted that this process is dependent upon Rad51C, a single-stranded DNA binding protein involved in supporting homologous recombination during DNA double-strand break repair (16). Therefore, it is possible that in the case of fAAV2, cellular DNA damage serves two purposes: (i) derepression of the single-stranded cassette by inhibitory proteins such as the MRN complex and (ii) recruitment of homologous recombination proteins such as Rad51C to facilitate the annealing of fragment vector DNA. Interestingly, treatment with bortezomib also resulted in high levels of fAAV2 enhancement. Treatment with proteasome inhibitors has been shown to increase the sheer volume of vector particles that reach the nucleus. It is possible that the deficit observed in unassisted fAAV2 transduction is simply a number

game; i.e., as more particles, and therefore presumably more uncoated genomes, transit to the nucleus, there is an increased chance for fragment strand reannealing. Alternatively, inhibition of the proteasome may also inhibit the degradation of proteins that might be essential for fAAV2 reannealing. Indeed, work by Bennett and Knight has shown that proteasome-mediated degradation of Rad51 occurs during DNA repair and this process is regulated in part by Rad51C (90). Additionally, the proteasome has been shown to be associated with double-strand breaks and has been suggested to play a role in degrading proteins upon completion of DNA repair in yeast (91). Regardless of the mechanism, our results suggest that the deficit in fAAV2 transduction can be restored to ssAAV2 levels through pharmacological intervention, thus paving the way for future studies in pharmaco-gene therapy for large gene applications.

While the compounds that induced DNA damage seemed to enhance rAAV transduction to the greatest extent, caution is necessary before potentially moving forward with any of these drugs. Beyond concerns of direct unwanted side effects of the various drugs (for example, etoposide has >30 undesired consequences), indirect altered vector maintenance should also be thoroughly analyzed. Though the primary mechanism of DNA persistence is through concatamerization and circularization, random integration has been demonstrated for rAAV (92–97). Indeed, a previous study has shown that integration of rAAV DNA *in vitro* increased upon treatment with etoposide (38, 98). While the epipodophyllotoxins have been shown to produce breaks in DNA "hot spots" (99–101), a thorough assessment of *in vivo* integration events would need to be carried out in order to gauge the risks of this kind of pharmaco-gene therapy. Finally, the effects of these agents on episomal expression should also be evaluated. It is tempting to envision a scenario in which inducing DNA damage through one of these agents could reactivate persistent-but-silenced rAAV episomes, thus eliminating the need for additional administration of vector if transgene expression falls below therapeutic levels.

## ACKNOWLEDGMENTS

Animal studies were performed within the LCCC Animal Studies Core facility at the University of North Carolina at Chapel Hill. The LCCC Animal Studies Core is supported in part by an NCI Center Core support grant (CA16086) to the UNC Lineberger Comprehensive Cancer Center. This work was supported by the Northwest Genome Engineering Consortium, the Jain Foundation, and the NIH (RO1AI072176-06A1, RO1AR064369-01A1). Funding was also provided in part through a departmental unrestricted grant from Research to Prevent Blindness, New York, NY.

Serum markers for toxicity were evaluated by the UNC Clinical Chemistry Core facility.

We thank Shophia Shih for determining all the rAAV titers used here.

## FUNDING INFORMATION

This work, including the efforts of Matthew L. Hirsch and R. Jude Samulski, was funded by HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) (RO1AI072176-06A1). This work, including the efforts of Matthew L. Hirsch, was funded by Research to Prevent Blindness (RPB). This work, including the efforts of Matthew L. Hirsch and R. Jude Samulski, was funded by HHS | NIH | National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) (R01AR064369).

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