Proteasome Inhibitors Enhance Gene Delivery by AAV Virus Vectors Expressing Large Genomes in Hemophilia Mouse and Dog Models: A Strategy for Broad Clinical Application

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Delivery of genes that are larger than the wild-type adeno-associated virus (AAV) 4,681 nucleotide genome is inefficient using AAV vectors. We previously demonstrated *in vitro* that concurrent proteasome inhibitor (PI) treatment improves transduction by AAV vectors encoding oversized transgenes. In this study, an AAV vector with a 5.6 kilobase (kb) factor VIII expression cassette was used to test the effect of an US Food and Drug Administration–approved PI (bortezomib) treatment concurrent with vector delivery *in vivo*. Intrahepatic vector delivery resulted in factor VIII expression that persisted for >1 year in hemophilia mice. Single-dose bortezomib given with AAV2 or AAV8 factor VIII vector enhanced expression on average ~600 and ~300%, respectively. Moreover, coadministration of AAV8.canineFVIII (1×10^{13} vg/kg) and bortezomib in hemophilia A dogs ($n = 4$) resulted in normalization of the whole blood clotting time (WBCT) and 90% reduction in hemorrhages for >32 months compared to untreated hemophilia A dogs ($n = 3$) or dogs administered vector alone ($n = 3$). Demonstration of long-term phenotypic correction of hemophilia A dogs with combination adjuvant bortezomib and AAV vector expressing the oversized transgene establishes preclinical studies that support testing in humans and provides a working paradigm to facilitate a significant expansion of therapeutic targets for human gene therapy.

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INTRODUCTION

The use of adeno-associated virus (AAV) vectors for correction of many clinically relevant congenital deficiencies has been hampered by the relatively limited packaging capacity imposed by the small size of the wild-type AAV genome (4,681 nt) and inefficient AAV vector delivery of genes that are larger than the wild-type (wt) AAV genome. AAV vectors for delivery of larger genomes (based on expression cassettes up to and exceeding 6.0 kb) have been produced; however, these vectors are inefficient, and multiple investigators have characterized their limitations.[1–6] Strategies to improve the expression of large transgenes using AAV vectors include splitting large genomes for co-delivery using >1 AAV vector, or exploiting head-to-tail concatemer formation.[5,7,8] Although these approaches fundamentally solve current packaging limitations of AAV, they create other disadvantages. Cells have to be infected with numerous virus particles to increase the probability of transduction, and the system is reliant upon the efficiency of homologous recombination. Using deleted transgenes that retain therapeutic potential[9] and optimizing expression cassettes with minimal transcriptional regulatory elements,[5,10] when possible, have emerged as alternative solutions.

An understanding of the steps that follow receptor-mediated internalization of AAV into target cells is emerging, including mechanisms of endocytic processing and nuclear trafficking.[3,11,12] These studies demonstrate that the majority of viral capsids do not appear to deliver their genome to the nucleus, and the current hypothesis suggests that AAV capsids are cleared from the cell by either of two major pathways for degrading cellular proteins, via the lysosome or the proteasome. In order for proteins to be degraded by the proteasome, they must first be conjugated to ubiquitin by specific cellular ligases. Previous research has demonstrated *in vitro* that AAV capsids can be ubiquitinated, but it is still unclear whether ubiquitination marks capsids for proteasomal degradation or acts as a trafficking signal. Proteasome inhibitors (PIs) are known to increase capsid ubiquitination and significantly enhance infection efficiency in a cell-type and serotype-specific manner. Although

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the exact mechanism of enhancement remains unknown, it has been postulated that PIs do one or more of the following activities: block degradation of capsids in the cytoplasm, increase trafficking efficiency, block degradation of capsids in the nucleus, or indirectly improve genome stability; recent studies demonstrate PIs may impact processing after uncoating (R. Jude Samulski, unpublished results). *In vivo*, PIs affect other cellular functions, *e.g.*, immune processing.[13–15] Among the available PIs, bortezomib (also called PS-341 or Velcade) is the only one approved for clinical use. Bortezomib inhibits proteasome activity by forming a covalent bond with the active site threonine in the 20S core of the 26S proteasome particle and inhibits the chymotryptic activity of the proteasome. Bortezomib has become especially important in the treatment of multiple myeloma, either as single-agent therapy or in combination with other drugs, including the corticosteroid dexamethasone.[16]

Hemophilia A, a genetic disorder caused by the deficient activity of the large coagulation protein factor VIII, has attracted significant attention as a target condition for gene therapy.[17] Three human clinical trials of factor VIII gene therapy have failed to achieve persistent phenotypic correction. These trials utilized *ex vivo* fibroblast transduction, retrovirus vector, or gutted adenovirus vector for FVIII gene delivery.[17] Previously Sarkar *et al.* reported that expression of a 5.6 kilobase (kb) coagulation factor VIII gene expression cassette delivered to the liver by serotype AAV8 vector was possible in mice and dogs, but the efficiency of infection was extremely low (~1 infectious particle: 40,000 total vector particles)[6] and limiting with respect to vector production, dose required for correction,[6,18] and the potential to translate to human clinical application. The potential for factor VIII expression as well as the inefficiency of the large transgene vector having been established by that study, we chose to examine the same vector and similar transduction conditions in the presence or absence of PI at the time of vector delivery. The phenotypic improvements demonstrated in this report, and additional incremental innovations in factor VIII expression shown in subsequent efforts of others,[5,18–21] emphasize that strategies to overcome the inefficient delivery of large transgenes using AAV could have significant clinical implications.

We have previously demonstrated that a variety of AAV serotype (1–6) vectors can be used to deliver large transgene expression cassettes and effectively transduce cells,[3] albeit with an efficiency that is about one log lower than vectors produced using genomes of wtAAV size (<4.7 kb). Recently, three studies have characterized in greater detail the limitations of packaging large genomes in AAV capsids (*e.g.*, vectors produced from oversize genomes have lower particle number, lower transduction efficiency, and inefficient expression *in vivo*).[22–24] These studies postulated complementation of incomplete genomes as a means to obtain protein expression.[25] Using *in vitro* cell culture studies, we determined that PI treatment concurrent with AAV delivery improves transduction by AAV vectors that have been generated with larger genomes to a greater extent than AAV generated using the wt-size genome, allowing them to overcome to some degree the inherent inefficiency of large transgene vectors.[3] Although the mechanism continues to be investigated, based on these *in vitro* observations, we propose this limiting aspect of oversized AAV vector transduction can be overcome pharmacologically *in vivo* by specifically using combination drug/vector therapy. In this report, we describe the details of combination therapy that utilizes limited exposure to bortezomib, a drug with well-defined preclinical and clinical toxicity and efficacy profile, to improve AAV-mediated expression of a large factor VIII transgene, achieving correction of hemophilia A mouse and canine models with follow-up of >2 years.

RESULTS

PI bortezomib dose *in vivo*

The primary objective of these studies was to validate combination therapy using a clinically utilized PI and AAV transgene vectors in small and large animal disease models. PIs have been reported to increase rAAV vector transduction *in vitro* and *in vivo* by several mechanisms.[3,11,12,26–28] Although we had previously studied several PIs in tissue culture,[3,27] we wished to examine bortezomib (also known as PS-341 or Velcade), as this is the only PI currently approved for human clinical use. Bortezomib, like most chemotherapy agents, is dosed based on body surface area. For all large animal studies, we used the US Food and Drug Administration–approved dose of 1.3 mg/m^2 (see http://www.accessdata.fda.gov/drugsatfda_docs/label/2008/021602s015lbl.pdf). This dose extrapolates to ~0.5–1.0 mg/kg of body weight for C57Bl/6 hemophilic mice weighing 25–30 g. Preclinical toxicity data have established a safe dose for frequent recurrent therapy of 0.5 mg per kilogram of body weight in mice;[29] in these studies, mice receiving 1.0 mg/kg twice a week for 4 weeks experienced changes in weight, activity, and level of consciousness. We independently established that mice given a single dose of 0.5 mg/kg without AAV vector tolerated the PIs without apparent side effect or change in behavior or hematologic parameters (data not shown). Therefore, bortezomib was given at 0.5 mg/kg by a single portal vein injection coadministered with the AAV vectors in all mice studies, and this total dose used in mice is roughly equivalent to the total dose/kg body weight used in dog studies described below.

PI effect on AAV serotypes carrying wild-type size factor IX expression cassette in hemophilia B mice

Our previous *in vitro* studies suggest that proteasomal processes limit large transgene expression more than expression from vectors delivering wtAAV size genomes. To evaluate this hypothesis, we tested the effect of combination therapy using bortezomib on AAV-mediated *in vivo* correction of hemophilic animals. We first used a vector carrying a genome approximately the same size as wild-type AAV for the correction of the hemophilia B mouse model. An AAV vector carrying a human factor IX expression cassette driven by the CMV enhancer/chicken β-actin promoter, described previously,[30] was infused via the portal vein to hemophilia B mice with or without a single dose of bortezomib at the time of the vector delivery. As shown in **Figure 1a**, ssAAV2. FIX-treated mice averaged 2.4% factor IX activity over 20 weeks of observation. Bortezomib treatment resulted in an average expression of 4.3% factor IX over the same period, equivalent to an 83% increase in factor IX expressed. Liver-directed factor IX expression was more efficient using AAV8 than AAV2, and mice

receiving vector alone expressed on average 40% factor IX activity over 20 weeks. Factor IX expression over 20 weeks was modestly augmented in mice receiving the single dose of bortezomib coadministered with the AAV8 vector, averaging ~24% higher factor IX activity (**Figure 1b**).

PI effect on AAV serotypes expressing larger than wild-type size cFVIII expression cassette in hemophilia A mice

Having determined that coadministration of bortezomib with AAV vector carrying wt size factor IX transgene cassette results in increase (although modest) of both AAV serotypes 2 and 8 transduction in the factor IX–deficient mouse model, we next focused on the effect of combination therapy using a large transgene (FVIII) cassette in hemophilia A mice of the same genetic background (C57Bl/6). The factor VIII cDNA (~7.1 kb) contains a large B domain (~2.7 kb) that does not contribute to blood clotting; once the majority of sequence coding for the B domain is removed, the cFVIII cDNA sequence of 4.5 kb can be incorporated in AAV vectors. Nevertheless, the addition of promoter and other required transcriptional regulatory elements to these vectors results in an oversized AAV expression cassette of 5.6 kb. AAV2 and AAV8 vectors were generated using the 5.6 kb FVIII expression plasmid. Virus was characterized for total particle number by qPCR, virion integrity by electron microscopy, capsid ratio by silver stain, and DNA genome size by alkaline gel electrophoresis (data not shown). For multiple production runs of vectors used for both mouse and canine studies, all these vector parameters were consistent with our previous published studies[3] and those of others.[6,22–24] For FVIII studies, the PIs MG-132 (also called Z-LLL) and bortezomib were chosen so that we could compare the most effective PI used in our previous report[3] (MG-132) with the clinically approved agent, bortezomib. Both MG-132 and bortezomib led to increased FVIII transgene expression compared to mice receiving vector alone (**Figure 1c,d**). As shown in **Figure 1c**, FVIII activity was undetectable until week 8 after AAV2 vector alone, whereas factor VIII was detectable at week 2 in mice receiving coadministration of MG-132 and at week 1 in mice that received bortezomib. Factor VIII expression was increased on average >150% by MG-132 and >550% by bortezomib.

In contrast to the delayed onset of expression observed in mice receiving AAV2 vector without PI, mice receiving the AAV8 vector alone (**Figure 1d**) had canine FVIII detectable at the earliest examination at week 1 after transduction. In these mice, canine factor VIII peaked at 8 weeks, then began to decrease. Although slightly increased factor VIII expression was observed in the first month after AAV8 with the coadministration of MG-132, the improvement of factor VIII expression was not significant or persistent, as was observed using bortezomib. Bortezomib coadministration improved initial and sustained canine factor VIII expression. On average, factor VIII expression was 259% higher in mice receiving the single dose of bortezomib when compared to mice receiving AAV8.cFVIII vector alone. Analysis of liver alanine aminotransferase and aspartate aminotransferase, and complete blood counts did not show any evidence of hepatic or hematologic toxicity in any mice receiving vector alone or in combination with bortezomib. To facilitate comparison of the PI effect upon vectors generated from wtAAV-sized factor IX transgene and oversized factor VIII transgene, the same time points are graphed in **Figure 1a,b** (factor IX) and **Figure 1c,d** (factor VIII). Bortezomib treatment was associated with a proportionately greater augmentation of the oversize transgene expression than that of the smaller transgene. This result provides *in vivo* corroboration of our *in vitro* studies using chloramphenicol acetyltransferase gene-CAT or CFTR transgene cassettes.[3]

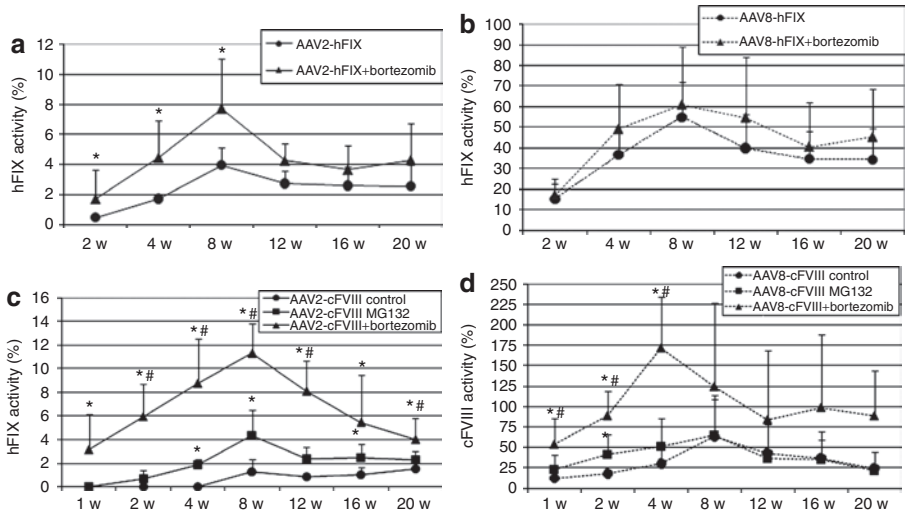

Figure 1 Effect of proteasome inhibitor combination therapy with AAV2 and AAV8 vectors for the correction of factor IX–deficient and factor VIII–deficient mice. Hemophilic mice received 3×10^{10} vector genomes/mouse of single AAV2 or AAV8 vectors with or without proteasome inhibitor (0.5 mg/kg body weight) by portal vein injection. (**a**,**b**) Percent of normal human factor IX activity in $FIX^{-/-}$ mice following AAV2 (**a**) or AAV8 (**b**) with or without bortezomib. (**c**,**d**) Percent of normal canine factor VIII activity (Coatest assay) in $FVIII^{-/-}$ mice following AAV2 (**c**) or AAV8 (**d**) in the presence of proteasome inhibitors MG-132 and bortezomib. Data are presented as mean ± SD. *Bortemozib or MG-132 versus AAV-cFVIII control, $P < 0.05$. #Bortemozib versus MG-132, $P < 0.05$, $n = 3$–4/group in **a**; $n = 5$–6/group in **b**; $n = 5$/group in **c**; $n = 6$/group in **d**. Statistics was performed by analysis of variance on SigmaStat software.

PI increases nuclear accumulation of genomes *in vivo*

PIs have been reported to increase rAAV vector transduction *in vitro* and *in vivo* by several mechanisms.[3,11,12,26,27] Recently, we described a study wherein rAAV2 virions enter the nucleus intact, are sequestered in the nucleolus in stable form, and can be mobilized from the nucleolus to nucleoplasmic sites likely permitting uncoating and subsequent gene expression or genome degradation. By examining these processes in the presence of PIs, we have refined our understanding of AAV2 trafficking dynamics and have identified cellular parameters that mobilize virions in the nucleus that significantly influence AAV infection. To evaluate trafficking dynamics *in vivo*, hemophilia A and B mice were next treated with combination therapy of PI, and either factor VIII or factor IX vector, respectively; the abundance of vector sequences in the cytoplasm and nuclear compartment were compared. Two weeks after vector administration with or without bortezomib, total liver cell cytoplasmic and nuclear fractions from AAV8.FVIII- or AAV8.FIX-treated mice were purified and examined to determine the intracellular location of vector genomes. The ratio of genomes persisting in the nucleus rather than cytoplasm was increased by the single dose of bortezomib, supporting a role for inhibition of the proteasome pathway in promoting nuclear translocation (**Table 1**). This effect was demonstrated for both the oversized transgene (factor VIII) vector as well as for the wild-type size (factor IX) vector. These results, consistent with our *in vitro* studies, suggest a pathway that may divert vector genomes from cytoplasm to nuclear structures for subsequent steps in vector uncoating and/or genome complementation as suggested by recent reports.[22–25] However, additional mechanism(s) likely exist to account for the augmented expression of oversized transgene vectors.

Effect of dexamethasone upon ectopic AAV vector expression during liver-directed gene therapy

Bortezomib and dexamethasone are commonly used together in clinical oncology therapies. Compared with bortezomib therapy alone, the combination of dexamethasone and bortezomib has increased efficacy, as has the addition of dexamethasone in the setting of incomplete clinical response to bortezomib monotherapy.[31–34] To determine whether a similar cooperative action between these agents might enhance vector transduction by oversized transgene cassettes, we evaluated outcome of vector transduction in multidrug combination therapy. For example, in previous studies, we had examined various additional pharmacologic agents in an effort to decrease the uptake of AAV vectors expressing reporter gene at ectopic sites during liver-directed gene therapy (**Supplementary Materials and Methods**). During these early studies, we observed a trend to decrease ectopic vector scatter when dexamethasone was coadministered with vector. This was reflected in lower gene expression and lower genome persistence in several organs; for example, mean expression of marker gene was decreased 10–30 times in heart, spleen, and pancreas (**Supplementary Table S1** and data not shown). The degree of interanimal variation in off-target expression in the absence of dexamethasone was large, so that the trend of the dexamethasone effect was not statistically significant. Based on the clinical use of dexamethasone with bortezomib, and results above, we chose to treat an additional group of AAV8.cFVIII/bortezomib mice simultaneously with a single dose of the corticosteroid dexamethasone and examine the effect upon persistent transgene expression (see below).

Table 1 Proteasome inhibitor increases nuclear accumulation of genomes *in vivo*

Treatment	Cytoplasm	Nucleus	Ratio N/C
AAV8-FIX	6.27 ± 1.47	3.21 ± 1.1	0.55 ± 0.25
AAV8-FIX+bortezomib	0.58 ± 0.43	13.2 ± 1.95	22.8 ± 27.3*
AAV8-cFVIII	4.46 ± 0.7	5.52 ± 0.96	1.23 ± 0.9
AAV8-cFVIII+bortezomib	1.2 ± 0.91	9.08 ± 2.61	7.57 ± 3.62*

Abbreviations: cFVIII, canine factor VIII; N/C, nuclear/cytoplasm.
Data are represented as mean ± SEM.
*$P < 0.05$.

Long-term observation of pharmacologic enhancement of AAV.cFVIII expression in FVIII knockout mice

Having established the potential of bortezomib to enhance factor VIII expression, additional mice were treated with AAV2 and AAV8 vectors to determine the duration of bortezomib enhancement. Circulating factor VIII activity in mice receiving AAV2 alone was first detectable at 8 weeks, and fluctuated around the lower limit of detection of this assay (~1% activity = 0.01 IU/ml) throughout 1 year of observation. (**Figure 2**) However, coadministration of bortezomib resulted in detectable factor VIII activity at week 1 in most mice, which peaked at week 8 (0.11 ± 0.06 IU/ml) and was maintained at >3% for a year. Expression of cFVIII from the AAV8 vector peaked at week 8 (0.51 ± 0.38 IU/ml) before gradually decreasing to a plateau ~10% of normal human factor VIII activity that was maintained to week 52 (the final observation, 0.10 ± 0.019 IU/ml). In contrast, mice treated with AAV8.cFVIII and the single dose of bortezomib exceeded even at the first time point (week 1) the peak value of the vector-only group, and maintained cFVIII activity of >50% normal throughout 1 year of observation (cFVIII activity 0.67 ± 0.58 IU/ml at week 52), as shown

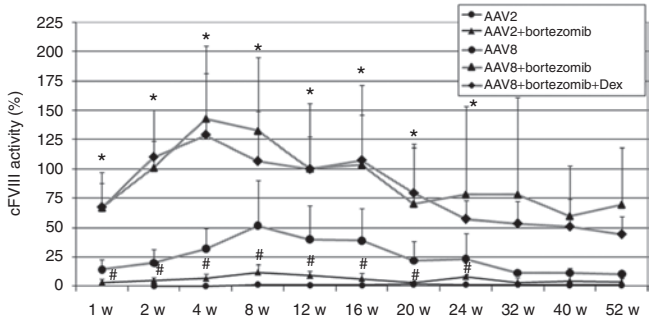

Figure 2 Persistent adjuvant effect using single-dose bortezomib upon AAV2- and AAV8-mediated cFVIII expression in hemophilia A mice. FVIII$^{-/-}$ mice were injected with AAV2 or AAV8 expressing cFVIII at the dose of 3×10^{10} gc/mouse, without proteasome inhibitor or with bortezomib (0.5 mg/kg body weight) or bortezomib plus dexamethasone (0.2 mg/animal, equivalent to 8 mg/kg body weight) by portal vein injection. Citrated plasma was collected at defined time points and FVIII activity determined by Coatest assay. Data are presented as mean ± SD % normal canine FVIII activity. *AAV8.cFVIII + bortezomib (with or without dexamethasone) versus AAV8.cFVIII vector alone, $P < 0.05$. #AAV2.cFVIII + bortezomib versus AAV2.cFVIII vector alone, $P < 0.05$.

in **Figure 2**. In these studies, the overall pattern of expression in bortezomib-treated mice was similar with and without the addition of dexamethasone.

Development of canine FVIII–neutralizing antibodies in FVIII knockout mice

As described in the original report using this canine factor VIII expression construct,[6] it is expected when treating factor VIII knockout mice that inhibitory antibodies against the factor VIII transgene may arise, given the cross-species origin of the protein. In our experience and in published reports,[35] even one intravenous exposure to 1.0 unit human FVIII protein may lead to antibody formation in this strain of mice. Anti-cFVIII inhibitor antibody formation was largely avoided in the earlier study when mice were pretreated with cyclophosphamide as well as hemostatic support with 1.2 U recombinant human FVIII for surgery.[6] As shown in **Table 2**, some mice in each of our treatment groups did develop FVIII-neutralizing antibodies at various times 2–20 weeks after vector exposure, despite cyclophosphamide dosing performed per the schedule outlined by Sarkar *et al.*[6] Inhibitor titers were near the lower limits of detection in the few AAV2.cFVIII-treated mice that developed antibodies, and the significance of these borderline titers is unclear. As previously reported by Jiang *et al.*,[19] inhibitors developed more frequently following AAV8.cFVIII than with AAV2.cFVIII, being seen in 6/10 mice receiving AAV8.cFVIII without PI. Coadministration of bortezomib appeared to decrease the incidence of inhibitor formation in animals treated with the AAV8 serotype vector (5/15 mice, 33.3%). The combination of corticosteroid and bortezomib was associated with the lowest rate of inhibitor formation (20% of AAV8.cFVIII/bortezomib/dexamethasone mice versus 60% of vector-only mice), suggesting potential benefit from this combination therapy.[36]

Phenotypic improvement of hemophilia A dogs given bortezomib concurrently with AAV vector

One primary object of this study was to validate combination therapy using bortezomib for AAV enhancement of oversized transgene in large animal model to better substantiate potential use in human. Using the identical AAV-cFVIII vector cassette used in the hemophilia A mouse studies, we next determined whether PI augmentation of the expression of large transgenes translates to the canine hemophilia A model. As a preliminary safety study, two hemostatically normal dogs were treated with a course of four doses of bortezomib using a treatment schedule typical for use in human oncology applications (1.3 mg/m^2 twice a week for 2 weeks). The dogs developed no clinical symptoms, blood chemistry, or cell count abnormalities (data not shown). Next, as shown in **Table 3**, a total of seven hemophilia A dogs were treated with AAV8.cFVIII with follow-up of at least 10 months (longest follow-up = 32 months). Three hemophilia A pups received a limiting dose of 1×10^{13} vg/kg AAV8.cFVIII (AAV8) via the portal vein. Four hemophilia A pups received the same AAV therapy and received a single i.v. dose of the PI bortezomib at the clinically approved dose of (1.3 mg/m^2). The vector and PI were well tolerated. Specifically, no liver transaminase elevations, neurologic symptoms, or changes in hematologic parameters occurred, and there was no development of factor VIII–neutralizing antibodies (FVIII inhibitors).

Correction of plasma clotting potential was quantified using a factor VIII–specific two-stage chromogenic factor VIII activity assay (Coatest) and the whole blood clotting time (WBCT). Peak factor VIII activity levels were achieved between days 7 and 21 after vector delivery. Peak factor activities ranged from 0.5 to 1.0 mIU/ml in two dogs receiving the AAV8.FVIII vector alone and was undetectable in the third dog. The factor VIII activity ranged from 2.2 to 3.8 mIU/ml in dogs receiving bortezomib with vector delivery; FVIII activity was not detected in one male dog that received vector alone and another female dog that received vector + bortezomib but without dexamethasone (**Table 3**). By the second month after vector delivery, only dogs that received bortezomib (± dexamethasone) maintained factor VIII activity above 1%, which the manufacturer defines as the level of sensitivity of the Coatest. To continue to allow relative comparisons of plasma clotting potential between the groups, all subsequent testing used the WBCT, as this assay detects the clotting potential of much lower factor concentrations. The WBCT in hemostatically normal dogs is 6–10 minutes; the WBCT in untreated hemophilia A dogs is >20 minutes. Examining the potential effect of combination therapy at the earliest time point, the mean WBCT at 1 week after delivery of AAV8 alone was >16 minutes, as compared to 8.8 minutes in dogs receiving bortezomib + AAV8 (**Figure 3** and **Table 3**).

Remarkably, the effect of the single dose of bortezomib was evident for months. Examining correction during the first 10 months following vector delivery (the minimum amount of follow-up that is available for all seven dogs), the two male dogs receiving AAV8 alone had only 4 of a total of 40 (10%) individual WBCT measurements in the normal range; males getting bortezomib + AAV8 had 33 of 44 (75%) individual WBCT assays completely normalized during the same period of observation. At longest follow-up (>11 months for all dogs), the male that received bortezomib + dexamethasone concurrent with AAV8 delivery was the only dog that maintained activity detectable in the chromogenic factor VIII activity assay (1.7–3.8 IU/ml at 33 months after vector). Liver transduction using AAV2 and AAV8 gene[10,37] therapy vectors has been reported previously to be less efficient in females than in males. Female dogs receiving bortezomib + AAV8 had 12 of 40 (30%) individual WBCT measurements corrected into the normal range over 10 months of follow-up; a female getting vector without PI had only 1 of 20 (5%) of WBCT values in the normal range.

Table 2 Anti-cFVIII inhibitor development after AAV2 and AAV8 treatment in FV1114 mice

Animals with inhibitor/total animals		Time of onset after vector (week)	Inhibitor titer (range)
AAV2-cFVIII	1/5	8	0.4–0.5 BIU
AAV2-cFVIII+bortezomib	2/6	4 and 8	0.4–1.2 BIU
AAV8-cFVIII	6/10	8–20	1.2–10.4 BIU
AAV8-cFVIII+bortezomib	5/15	2–20	1.0–13.2 BIU
AAV8-cFVIII+bortezomib+Dex	2/10	6 and 20	0.5–2.4 BIU

Abbreviations: BIU, Bethesda units; cFVIII, canine factor VIII; Dex, Dexamethasone.

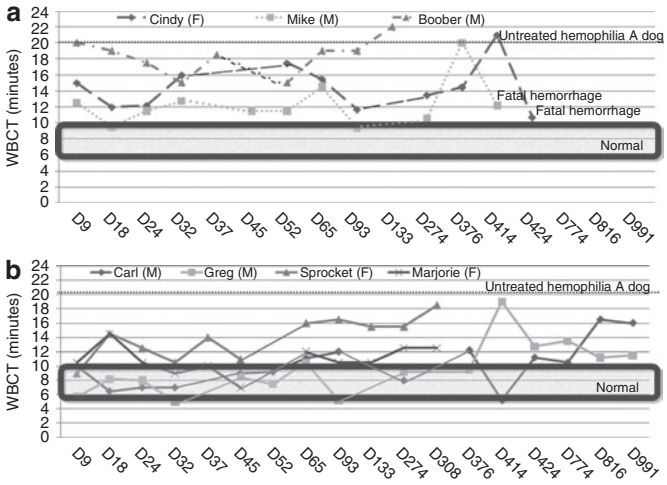

Figure 3 Persistent correction of hemophilia phenotype by adjuvant proteasome inhibitor with liver-directed AAV8-mediated cFVIII gene therapy in hemophilia A dogs. (**a**) Whole blood clotting time (WBCT) in two male and one female hemophilia A dogs treated with vector only and no bortezomib. (**b**) WBCT in two male and two female hemophilia A dogs treated with coadministration of a single dose of i.v. bortezomib at the time of AAV vector administration. Normal range of 6–10 minutes for hemostatically normal dogs is indicated by the shaded region. All hemophilic dogs had baseline WBCT >20 minutes.

Importantly, the combination therapy augmentation of plasma clotting potential translated directly into correction of the bleeding phenotype (**Table 3**). This strain of hemophilia A dogs typically experiences bleeds about six times/year. Three age-matched untreated hemophilic dogs from the same colony bled 10, 10, and 13 times each during 22 months' observation concurrent with this study, resulting in a monthly bleeding rate of 0.50 ± 0.08 hemorrhages/month. The dogs receiving AAV8.FVIII vector alone had a mean bleeding frequency of 0.80 ± 0.26 bleeds/month, not significantly different from untreated hemophilic dogs ($P = 0.23$). Dogs that received bortezomib with vector had a mean of 0.07 ± 0.08 hemorrhages/month, which was a striking improvement compared to matched, untreated hemophilic dogs ($P < 0.001$) and vector-only treated dogs ($P < 0.003$). The original treatment group ($n = 4$) have now been followed for >32 months. Bortezomib + AAV8.cFVIII combination therapy dogs have had one and two spontaneous bleeds, respectively. During the same period, the dogs that received only vector had 12 and 16 bleeds before each experiencing a fatal hemorrhage at 16 and 25 months after vector treatment, respectively.

DISCUSSION

Correction of hemophilia A through gene therapy remains an elusive goal of modern medicine. Strategies using AAV vectors are attractive, but expression of factor VIII has thus far been inefficient, in part, because the factor VIII cDNA exceeds the packaging capacity of wt AAV. We have previously demonstrated that AAV vectors carrying oversize transgene cassettes transduce cells less efficiently.[3] More importantly, this inefficiency can be overcome by the use of pharmacologic agents such as PIs.[3] Based on these observations, we hypothesized that concurrent administration of the PI bortezomib with an AAV vector may have an adjuvant effect to facilitate persistent correction of diseases requiring expression of a large transgene. Here, we demonstrate that the expression of an oversized factor VIII cDNA delivered by AAV can be enhanced several fold in hemophilic mice and dogs. The enhanced expression was demonstrated using two different serotype AAV vectors. The effect persisted *in vivo* without further proteasome treatment (>1 year in hemophilic mice and >2-1/2 years in hemophilic dogs). Most importantly, in the hemophilia A dogs, the single-dose PI therapy safely augmented a limiting AAV vector dose, which did not protect against hemorrhagic complications of the hemophilic phenotype in the absence of drug. As a result, only the dogs receiving bortezomib "combination therapy" demonstrated persistent protection from the chronic recurrent and lethal bleeding complications of severe factor VIII deficiency.

Overcoming AAV barriers

Understanding the cellular pathways that facilitate this nonpathogenic DNA virus to carry out successful infection has been instrumental in AAV vector development. For example, a number of groups[11,38–40] have illustrated that drugs/chemicals such as bafilomycin A, brefeldin A, and MG-132 augment rAAV transduction by acting at the level of endosome acidification, early-to-late endosome transition, and proteasome activity, respectively. The ability of PIs such as MG-132 and LLnL to increase vector transduction has provided further support to the notion that proteasomes in the cytoplasm act as barriers to AAV infection. These barriers have been characterized in detail both *in vitro* and *in vivo*, and investigations of working models for mode of action are ongoing. For example, Duan *et al.*[12] described a tenfold increase in the proportion of hepatocytes expressing AAV2 alkaline phosphatase transgene after administration of PIs LLnL or Z-LLL supporting the hypothesis that viral capsid ubiquitination and subsequent proteasomal processing place limitation on AAV transduction. This study and subsequent studies from Engelhardt and others have confirmed the influence of the ubiquitin-proteasome system, also demonstrating this system affected additional AAV serotypes (*e.g.*, AAV5), but that the relative importance of this system differed between specific tissues.[11,12,26,39] Additional insight into the relative importance of capsid ubiquitination as a limitation to AAV gene delivery comes from a series of studies from Srivastava *et al.*[41,42] Pursuing the concept that phosphorylation of the AAV capsid (*e.g.*, by EGFR-PTK) is critical for subsequent ubiquitination, site-directed mutagenesis of surface-exposed tyrosines on the AAV capsid was performed to remove potential sites of phosphorylation. The tyrosine mutant AAV capsids were relatively resistant to proteasome-mediated degradation and demonstrated increased expression in *in vitro* and *in vivo* experiments when compared to tyrosine-phosphorylated AAV. These and other models are being actively pursued to better understand barriers to AAV transduction imposed by the ubiquitin/proteasome system.

Impact of PIs on AAV oversized transgene expression

In this study, we aimed to advance these insights to preclinical models using combination therapy with drugs having well-characterized toxicity and efficacy profiles (*e.g.*, PI bortezomib and corticosteroid dexamethasone) that would justify testing in human gene therapy trials. The current study confirms, in relevant

animal disease models, our previous *in vitro* observation that PIs overcome, in part, the inherent inefficiency of expressing large therapeutic transgenes from AAV. Our data strongly support the use of bortezomib with oversized AAV transgene cassettes such as FVIII. Although bortezomib is commonly given in combination with the corticosteroid dexamethasone, we did not observe in mice significant synergy in regards to enhancing hepatic gene expression when compared to bortezomib alone (**Figure 2**). Nevertheless, there may be additive effects upon antigen presentation and immunologic tolerance that justify combining these drugs in gene transfer applications, as the combination led to the lowest inhibitor antibody formation in the inhibitor-prone mice. (**Table 2**) Additionally, although the numbers of dogs treated do not allow statements regarding the significance of different treatment effects, greatest persistence of factor VIII activity was observed, for both the dogs of male and female gender, in the dogs that received the combined adjuvant drugs dexamethasone and bortizomib with AAV (Greg and Marjorie in **Table 3**).

In addition to the rescuing of oversized AAV transgene cassette transduction with the use of adjuvant bortezomib therapy, a more modest (less than twofold) augmentation was observed for wild-type size AAVFIX vectors (**Figure 1**). It is not clear why we see a more dramatic impact *in vivo* upon transduction of oversized genomes. In our previous *in vitro* study, AAV1 vectors encapsidating transgene cassettes of 4.7, 5.3, and 6.0 kb were used to transduce HeLa cells, and coadministration of the PI LLnL increased expression 1.1-, 2.2-, and 5.7-fold respectively, when compared to the level of expression each vector achieved in the absence of PIs.[3] Further analysis *in vitro* using bortezomib (see **Supplementary Figure S1**) demonstrated a tenfold increase of fluorescence on a single-strand wild-type size AAV2.GFP vector.

With respect to mechanism(s) that might explain the proportionately greater increase in expression by combining PI with oversized transgenes, as compared to wild-type size transgenes, it should be noted that proteasomes are present in both the cytoplasm and the nucleus. Although we demonstrate that nuclear accumulation is enhanced by bortezomib, it is possible that the relative enhancement of large transgene expression by PI may involve additional processes that occur in the nuclear compartment. For example, we have recently reported that AAV2 virions enter the nucleus and accumulate in the nucleolus after infection, whereas empty particles are excluded from nuclear entry.[27] The virions that accumulate at the nucleolus retain infectivity. Furthermore, PIs potentiate nucleolar accumulation, as demonstrated using AAV carrying wild-type size sequences. Nuclear trafficking, nuclear accumulation, and subsequent mobilization of the nucleolar fraction have not been studied using oversized AAV vectors, especially with regards to the relative effect of a PI on these mechanisms. More importantly, three papers recently described rigorous analysis of large genome encapsidation in AAV capsid. These studies independently demonstrated the propensity of AAV to package genomes efficiently within the range of 106% of wild-type size.[22–24] Fragmented vector genomes were encapsidated from transgenes above this size range (*i.e.*, cFVIII-5.6 kb). Similar observations have been documented for other viruses (*e.g.*, adenovirus packages between 88 and 105% of wild type) suggesting a "head-full" packaging mechanism governing viral DNA genome size. It is hypothesized that full-length protein expressed from these vectors results from "intracellular reassembly of partial gene fragments." It remains to be seen whether PIs facilitate a step in this newly hypothesized process of vector-fragmented genomes reassembly,[25] but it is tempting to speculate that concentration of virion genomes in nuclear compartments after PI treatment (as previously described by Johnson *et al.*[27]) would potentiate this possibility.

Proteasome inhibition and impact on AAV vectors carrying a variety of different genome configurations should lead to better understanding of how to exploit pharmaceuticals as adjuvants to

Table 3 Summary of hemophilia A dogs undergoing AAV-mediated gene therapy with proteasome inhibitor

					WBCT assays		Bleeding frequency			
Dog	Sex	Age[a]	Treatment	Peak FVIII activity (IU/ml)	No. of assays	No. of values corrected to normal range (% of total assays)	1st Year (no.)	Total (no./time)	Inhibitor status (BIU)	Outcome
Cindy	F	27 days	AAV8	0.5	26	1 (4%)	9	12/18 months	0	Fatal bleeding
Mike	M	22 days	AAV8	1.0	26	4 (15.4%)	9	16/25 months	0	Fatal bleeding
Boober	M	23 days	AAV8	ND	18	0 (0%)	NA	11/10 months	0	Alive
Carl	M	25 days	AAV8+bortezomib	3.1	34	20 (58.8%)	0	1/32.5 months	0	Alive
Greg	M	25 days	AAV8+bortezomib/ Dex	3.8	34	20 (58.8%)	0	2/32.5 months	0	Alive
Sprocket	F	23 days	AAV8+bortezomib	ND	20	4 (20%)	NA	2/11 months	0	Alive
Marjorie	F	23 days	AAV8+bortezomib/ Dex	2.2	20	8 (40%)	NA	0/11 months	0	Alive
Denver	M		No treatment	NA	NA			10/22 months	NA	Alive
Bass	M		No treatment	NA	NA			13/22 months	NA	Alive
Gator	M		No treatment	NA	NA			10/22 months	NA	Alive

Abbreviations: BIU, Bethesda units; Dex, with dexamethasone; NA, not applicable; ND, not detected; WBCT, whole blood clotting time.
Normal value of 6–10 minutes for WBCT. WBCT is expressed as number of measurements in normal range in the total measurements.
[a]Age at vector administration.

increase the action of gene vectors in "combination therapy." For example, when characterizing AAV vectors having either a conventional single-strand (ssAAV) or a self-complementing genome structure (scAAV), we observed an increase in the percentage of cells expressing transgene after AAV2.GFP transduction and bortezomib coadministration (see **Supplementary Materials and Methods** and **Supplementary Figure S1**). *In vitro*, the amount of fluorescence from the population of successfully transduced cells was augmented to an even greater degree (from a mean fluorescence index of 75–890, **Supplementary Figure S1**) than the augmentation we observed *in vivo* using ssAAV with WT-size FIX genome. The bortezomib *in vitro* augmentation of expression from a self-complementing vector was almost identical to that seen with ssAAV suggesting a common pathway regardless of vector template conformation. Interestingly, our scAAV *in vitro* analyses are consistent with the report by Nathwani *et al*. who documented an increase in expression of factor IX mediated by bortezomib when coadministered with a self-complementary AAV8.FIX vector.[28] Although there appear to be additional factors at play when augmenting oversized vector genomes compared to single-stranded and self-complementary AAV templates, we were able *in vitro* to extend the effect of bortezomib enhancement to lentiviral vectors (see **Supplementary Figure S1**), suggesting that this drug augmentation may have universal application with respect to viral vector gene transfer even though it may be working by multiple mechanisms.

"Combination therapy" and clinical trials

Preclinical licensure studies and extensive clinical experience with bortezomib and dexamethasone exist to suggest considering the use of these drugs in combination with gene delivery to augment persistent gene expression. It is important to recognize that both drugs have significant side effect profiles, although for each drug many of the side effects arise in a cumulative fashion, as each drug is usually dosed repeatedly in clinical applications. Single-dose dexamethasone may have potent short-term anti-inflammatory effects, and is at times given clinically to achieve anti-inflammatory effect even in the presence of bacterial or viral infectious processes. Clinically, cumulative bortezomib (generally given in multiple courses, each course consisting of 2 weeks of twice-weekly dosing) is associated with peripheral neuropathy, cytopenias (thrombocytopenia, neutropenia) and other toxicities, which may be severe. Single-dose toxicity in animal preclinical testing was minimal at the doses used in our study and approved for drug licensure. Nevertheless, the drug has a narrow margin of safety and liver failure as an idiosyncratic reaction can be seen at any time during treatment including with the first dose. Studies are ongoing in our laboratories to determine whether using lower doses of the single coadministration of bortezomib will also augment transduction while increasing the safety of our single adjuvant dose approach.

Undoubtedly, PIs can affect a multitude of cellular processes, and further studies are required to dissect the significant differences between oversized and wild-type AAV transgene cassettes. The specific application of PIs to enhance the delivery of transgenes that exceed the size of the wild-type AAV genome has not been demonstrated previously *in vivo.* Furthermore, until these studies, phenotypic improvement achieved by coadministration of a PI with AAV vector has not been demonstrated in any large animal model using AAV vectors. Nevertheless, the clear augmentation of AAV8-mediated expression of the large FVIII transgene that we report here using bortezomib, along with previous studies in which PI augment AAV1 and AAV5 vector transduction,[3,26] suggest that PIs may enhance the efficiency of AAV gene delivery in a wide variety of contexts.

In conclusion, we demonstrate in the context of multiple AAV serotypes, and in small and large animal disease models the beneficial safety profile and efficacy of the PI bortezomib to enhance AAV-mediated expression of large therapeutic genes in combination therapy. These findings warrant further investigation of mechanisms for improved vector transduction and establish preclinical parameters that support eventual testing in phase I clinical application.

MATERIALS AND METHODS

AAV vectors, HIV-based vectors, and additional drugs. The factor VIII expression vector used in these studies has been described previously by one of the authors (R.S.).[6] The vector contains a canine B domain deleted (BDD) FVIII (cFVIII) cDNA driven by a synthetically derived short liver-specific promoter/enhancer, followed by a chimeric intron (IGBP/enh/intron). The factor IX vector has been described previously and contains a 4.2 kb expression cassette including the hFIX cDNA (1.4 kb) under transcriptional control of CMV enhancer/chicken β-actin promoter (rAAV-CBA-hFIX)[30] All vectors were produced and titered at the UNC Virus Vector Core Facility as described previously,[43] including single-strand and self-complementary AAV2 encoding green fluorescent protein (GFP), driven by CMV promoter (total size about 1.5 kb); firefly luciferase encapsidated with AAV8 capsid; driven by CBA promoter; and AAV2 and AAV8 expressing human factor IX (hFIX). The HIV-based, integrase-deficient vector was generated by the transient transfection method as previously described.[44] Bortezomib (Millennium Pharmaceuticals, Cambridge, MA) was diluted in phosphate-buffered saline for injection. MG-132 (Calbiochem, La Jolla, CA) was dissolved with 70% ethanol to 20 mmol/l as stock solution. Dexamethasone was purchased from Sicor Pharmaceuticals, Irvine, CA.

Animal care and studies

Mice: FVIII-deficient mice ($FVIII^{-/-}$) with a targeted deletion of exon 16 of the FVIII gene[45] were generously supplied by H.H. Kazazian Jr.'s laboratory (University of Pennsylvania, Philadelphia, PA). C57Bl/6 $FIX^{-/-}$ mice have a targeted deletion of the promoter through the third exon of the FIX gene.[46] $FVIII^{-/-}$ and $FIX^{-/-}$ mice were bred in house. Wild-type C57Bl/6 mice for luciferase vector delivery at 7–8 weeks of age were purchased from the Jackson Laboratory (Bar Harbor, ME). Hemophilic mice were anesthetized using 2.5% Avertin for all procedures. All plasma samples were collected from the retro-orbital plexus into 3.2% sodium citrate and stored at −80 °C. Retro-orbital blood collection and bioluminescence imaging were performed under isoflurane anesthesia. All studies in mice were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

$FIX^{-/-}$ mice received portal vein injection of 3×10^{10} gc/animal AAV2.hFIX or AAV8.hFIX vector in a total volume of 200 µl with or without a single dose of bortezomib (0.5 mg/kg) at the time of AAV delivery. $FVIII^{-/-}$ mice underwent portal vein injection of 3×10^{10} gc/mouse AAV2.cFVIII or AAV8.cFVIII, along with bortezomib or MG-132 (0.5 mg/kg body weight), as well as mice received a single dose of 1 mg/kg of recombinant human factor VIIa (NovoSeven, Novo Nordisk, Bagsværd, Denmark) to support perioperative hemostasis. To avoid anticipated antibody-mediated immune responses to the canine FVIII transgene, all $FVIII^{-/-}$ mice received cyclophosphamide (Sigma Aldrich, St Louis, MO) injected intraperitoneally in three doses of 100 µg each on days −3, 0, and

+3, as previously described.[6] C57Bl/6 mice received 10^{11} vector genomes/mouse AAV8 expressing firefly luciferase under transcriptional control of CMV enhancer/chicken β-actin promoter via tail vein; groups receiving dexamethasone therapy were treated with 0.2 mg/animal intraperitoneally 1–2 hours prior to AAV delivery.

Animal care and studies

Hemophilia A dogs: The hemophilia A dogs were mixed-breed dogs from the hemophilia A colony initially housed at the Scott-Ritchey Research Center, Auburn University but now at the UAB Medical School. All studies in dogs were approved by the Institutional Animal Care and Use Committees of Auburn University and the University of Alabama, Birmingham, AL. All animals were housed in facilities that are AAALAC accredited. Treated dogs were males (n = 4) or females (n = 3) with severe hemophilia A. All seven dogs were administered AAV8.cFVIII by mesenteric vein administration 3–4 weeks following birth.[47] Four dogs (two females/two males) were also administered bortezomib (1.3 mg/m^2) at the time of vector administration. One male and one female dog were administered i.v. dexamethasone (1.0 mg/kg) at the time of vector and bortezomib administration.

Coagulation factor VIII and factor IX activity assays and canine factor VIII Bethesda inhibitor antibody assay in mice. Canine FVIII activity in mouse plasma was measured by the Coatest SP4 kit (Chromogenix, DiPharma, West Chester, OH) following the manufacturer's instruction with modification. Normal canine plasma (regarded as 100 percent activity = 1 IU/ml) was serially diluted into FVIII$^{-/-}$ pooled mouse plasma to generate the standard curve. Neutralizing antibodies to canine FVIII in mouse plasma were measured by the Bethesda assay using a STart 4 Coagulation Analyzer (Diagnostica Stago, Asnières, France) as described.[48] Human factor IX was measured based on one-stage factor IX activity assay (FIX-specific aPTT) as previously described, using a STart 4 Coagulation Analyzer.[49]

FVIII activity, coagulation, and Bethesda assays in dogs. Blood samples were obtained from normal controls, untreated FVIII controls, and treated hemophilia A dogs as described.[47] WBCT, FVIII activity, and Bethesda titer were measured as previously reported. The WBCT assay was terminated at 20 minutes if a clot had not formed.

Preparation of hepatic cell nuclear and cytoplasmic extract. Nuclear and cytoplasmic fractions were isolated as described[50] with slight modification. Mouse livers were perfused with phosphate-buffered saline, minced on ice and homogenized [buffer: 250 mmol/l sucrose, 50 mmol/l Tris-HCl (pH 7.5), 25 mmol/l KCl, 5 mmol/l $MgCl_2$, 0.5% NP-40, 1 mmol/l phenylmethylsulfonyl fluoride], using a Dounce homogenizer. Nuclei and other organelles were collected by centrifugation for 10 minutes at 3,000 rpm in a Sorvall clinical centrifuge. The supernatant was filtered using a 40 µm pore-size filter and used as the cytoplasm. The multilayered pellet was processed further into nuclear extract dissolved into distilled water.

SUPPLEMENTARY MATERIAL

Figure S1. Proteasome inhibitor increases expression of AAV-2 vectors *in vitro*.

Table S1. Effect of dexamethasone coadministration with AAV8.luciferase upon transgene expression outside of liver.

Materials and Methods.

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